PHARMACOKINETIC STUDY OF ANTI-TB DRUG *PARA*-AMINOSALICYLIC ACID AND ITS METABOLITES: A POSSIBLE RELATIONSHIP WITH THE DEVELOPMENT OF TOXICITY

Kim Adams

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Supervisor:

Prof H Reuter

Professor and Head of Division of Clinical Pharmacology

Co-supervisor:

Dr M Stander

Central Analytical Facilities, Mass Spectrometry Unit, Department of Biochemistry

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my original work and that I have not previously submitted it, in its entirety or in part, at any other university for a degree.

Signature:…….. Date:…….

ABSTRACT

Background

Para-aminosalicylic acid (PAS) is a bacteriostatic anti-tuberculosis (anti-TB) drug, used in the treatment of multi-drug resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB). While PAS has shown great efficacy, it is notorious for its association with gastrointestinal (GI) intolerance. The intolerance was considered to be due to the dosing strategy, which therefore caused clinicians to opt for divided doses as opposed to a single large dose daily, but no evidence of improvement has been reported. It has thus been proposed that the rate of absorption and/or metabolism of PAS could possibly be responsible for poor tolerability of the drug.

Aims

The aim of the study was to investigate the potential association between the plasma concentrations of the main metabolites, acetyl-PAS (APAS) and glycine-PAS (GPAS), and the occurrence of GI intolerance, after the administration of a granular slow release PAS (GSR-PAS) formulation.

Study design and methodology

A two-period study of PAS and its metabolites was conducted in 29 adult patients (≥ 18 years old) MDR- and XDR-TB, at the Brooklyn Chest Hospital, Cape Town, South Africa. These patients were assigned to a 4g twice-daily GSR-PAS regimen for 1 week followed by another week given 8g once-daily GSR-PAS. Whole blood was collected in EDTA-containing tubes at 1, 2, 3, 4, 6, 8, 12 and 24 hours post dose, at the end of each week.

PAS plasma concentrations were determined using an ultra high performance liquid chromatography system coupled to a tandem mass spectrometer, (UHPLC-MS/MS) of which the assay was developed and partially validated according to the Food and Drug Administration (FDA) criteria. Thereafter, pharmacokinetic analysis was performed for the metabolites and parent drug of which pharmacokinetic parameters C_{max}, AUC_{0-24} and T_{max} were determined by non-compartmental analysis, using Winnonlin software version 8.0.

The tolerability of PAS was evaluated using a 10-point visual analogue scale (VAS) rating the severity of the adverse effects (AE's) experienced, with the most left indicating no symptoms and the most right indicating severe symptoms. The patients completed the evaluation on a daily basis after dosing. The AE's evaluated included nausea, bloating, diarrhoea, vomiting, and abdominal pain and cramps.

A correlation analysis using STATA software version 15.1 was used to measure the association between each AE and the median C_{max} of PAS, APAS and GPAS using the Spearman's rank correlation. Statistical significant was set at a *P* value less than 0.05.

Results:

The developed method proved successful in the assay of APAS, GPAS and PAS in a single run of 4 minutes. A large inter-individual variability was observed in the C_{max} ranging from 40.42 to 102.41 mg/L for PAS, and 10.00 to 22.36 mg/L and 3.90 to 9.45 mg/L for APAS and GPAS, respectively. The VAS data reported that 26 patients had evidence of GI intolerance, but the majority of these scores were clustered around zero. Abdominal pain and cramps were found to be statistically more frequent in the 4g twice-daily than 8g once-daily regime [0.14(0 – 0.59) versus 0(0 – 0.08); median (IQR); p= 0.018]. Statistically significant inverse associations were observed between APAS

concentrations and bloating (rho= -0.448; p= 0.025) and diarrhoea (rho= -0.407; p= 0.044), respectively, for the twice-daily dose. The same inverse association was found for GPAS concentrations and diarrhoea (rho= -0.412; p= 0.041).

Conclusions:

Plasma concentrations of metabolites APAS and GPAS did not correlate with the occurrence of AE's. On the contrary, the data showed that higher plasma concentrations of APAS and GPAS were associated with lower scores of AE's, which were statistically significant relationships but considered clinically negligible. Further work with a larger population size may be needed to determine the true effect of metabolite formation on the presence of GI discomfort when treated with PAS.

OPSOMMING

Agtergrond

Para-aminosalisielsuur (PAS) is 'n bakteriostatiese anti-tuberkulosemedisyne wat in die behandeling van middelweerstandige (MDR) en uiters middelweerstandige (XDR) tuberkulose (TB) gebruik word. Alhoewel PAS aanmerklike doeltreffendheid toon, is dit bekend vir 'n verband met gastro-intestinale (GI) intoleransie. Die intoleransie word aan die doseringstrategie verbind, wat dus meebring dat klinikusse verdeelde dosisse verkies teenoor 'n enkele groot daaglikse dosis, maar geen bewyse van verbetering is egter gerapporteer nie. Daar is dus voorgestel dat die tempo van absorpsie en/of metabolisme van PAS moontlik vir swak toleransie van die medisyne verantwoordelik is.

Doelwitte

Die doel van die studie was om ondersoek in te stel na die moontlike verband tussen die plasmakonsentrasies van die hoofmetaboliete, asetiel-PAS (APAS) en glisien-PAS (GPAS), en die voorkoms van GI-intoleransie ná toediening van 'n formulering van 'n granulêre stadigvrystellende PAS (GSR-PAS).

Studie-ontwerp en -metodologie

'n Tweetydperk-studie van PAS en sy metaboliete is uitgevoer onder 29 volwasse MDR- en XDR-TB-pasiënte (≥ 18 jaar oud) by die Brooklyn Chest Hospitaal in Kaapstad, Suid-Afrika. Hierdie pasiënte is ingedeel vir 'n GSR-PAS-regimen van 4 g twee keer per dag vir een week, gevolg deur nog 'n week waarin hulle een keer per dag 8 g GSR-PAS ontvang het. Heelbloed is in EDTA-bevattende buise teen 1, 2, 3, 4, 6, 8, 12 en 24 ure ná toediening van die dosis aan die einde van elke week geneem.

PAS-plasmakonsentrasies is bepaal met behulp van 'n ultrahoëverrigting-vloeistof-chromatografiestelsel gekoppel aan 'n tandem-massaspektrometer, waarvan die toets volgens kriteria van die Amerikaanse Voedsel- en Medisyne-administrasie ontwikkel en bekragtig is. Daarna is farmakokinetiese ontleding vir die metaboliete en moedermedikasie uitgevoer, waarvan farmakokinetiese parameters C_{max}, AUC_{0-24} en T_{max} deur niekompartementele ontleding bepaal is met gebruik van Winnonlin-sagteware weergawe 8.0.

Toleransie van PAS is met behulp van 'n tienpunt- visuele analoë skaal (VAS) geëvalueer, wat die erns van die negatiewe gevolge (NG's) wat ervaar is, gerangeer het, met heel links wat geen simptome aantoon en heel regs wat ernstige simptome aantoon. Die pasiënte het die evaluering daagliks ná dosering voltooi. Die NG's wat geëvalueer is, het naarheid, opgeblaasdheid, diarree, vomering en maagpyn en -krampe ingesluit. 'n Korrelasie-ontleding met behulp van STATA-sagteware weergawe 15.1 is gebruik om die verband tussen elke NG en die mediaan C_{max} van PAS, APAS en GPAS aan die hand van die Spearman-rangkorrelasie te meet. Statisties betekenisvol is gestel as 'n *P*-waarde onder 0.05.

Resultate:

Die ontwikkelde metode het sukses getoon in die toets van APAS, GPAS en PAS in 'n enkellopie van 4 minute. 'n Groter interindividuele veranderlikheid is waargeneem in die C_{max}, wat gewissel het van 40.42 tot 102.41 mg/L vir PAS, en 10.00 tot 22.36 mg/L en 3.90 tot 9.45 mg/L vir onderskeidelik APAS en GPAS. Die VAS-data het getoon dat 26 pasiënte bewyse van GI-intoleransie getoon het, maar die meerderheid van hierdie tellings het trosvorming om nul getoon. Maagpyn en -krampe is gevind om statisties meer gereeld voor te kom by die regimen van 4 g twee keer per dag as dié van 8 g een

keer per dag [0.14(0 – 0.59) teenoor 0(0 – 0.08); mediaan (IQR); p = 0.018]. Statisties betekenisvolle inverse verbande is waargeneem tussen onderskeidelik APAS-konsentrasies en opgeblaasdheid (rho = -0.448; p = 0.025) en diarree (rho = -0.407; p = 0.044) vir die dosis wat twee keer per dag toegedien is. Dieselfde inverse verband is gevind vir GPAS-konsentrasies en diarree (rho = -0.412; p = 0.041).

Gevolgtrekkings:

Plasmakonsentrasies van metaboliete APAS en GPAS het nie ’n verband getoon met die voorkoms van NG’s nie. In teendeel, die data het getoon dat hoër plasmakonsentrasies van APAS en GPAS verband gehou het met laer tellings NG’s, wat statisties betekenisvolle verhoudings was, maar as klinies onbeduidend geag is. Verdere studie met ’n groot populasiegrootte is moontlik nodig om die ware uitwerking van metabolietvorming op die teenwoordigheid van GI-ongemak tydens behandeling met PAS te bepaal.

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LIST OF ABBREVIATIONS and SYMBOLS

≤	Less than or equal
≥	More than or equal
µg/mL	Microgram per millilitre
5-ASA	5-aminosalicylic acid
ADME	Absorption, distribution, metabolism and excretion
AEs	Adverse effects
AKN	Amikacin
AMX	Amoxicillin
APAS	*N*-acetyl-para-aminosalicylic acid (Acetyl-PAS)
API	Atmospheric pressure ionization
ARV	Antiretroviral
ASA	Aspirin (acetylsalicylic acid)
AUC	Area under the curve
BMI	Body mass index
CFM	Clofazimine
CFU	Colony forming units
CID	Collision induced dissociation
CL	Clearance
CLS	Cycloserine
C_{max}	Maximum concentration
C_{min}	Minimum concentration
CPX	Ciprofloxacin
CSF	Cerebrospinal fluid

CTM	Clarithromycin
CYP	Cytochrome P450
DHF	Dihydrofolate
DHP	Dihydroteroate
DHPS	Dihydroteroate synthase
DOTS	Directly observed treatment strategy
DR-TB	Drug resistant tuberculosis
DST	Drug susceptible testing
dTMP	Deoxythymidine 5'-monophosphate
dUMP	Deoxyuridine 5'-monophosphate
EBA	Early bactericidal activity
EDTA	Ethylendiaminetetraacetic acid
EFV	Efavirenz
ESI	Electrospray ionisation
ETH	Ethionamide
ETM	Ethambutol
F	Bioavailability
FDA	Food and Drug Administration
GC	Gas chromatography
GERD	Gastroesophageal reflux disease
GIT	Gastrointestinal tract
GPAS	*p*-Aminosalicyluric acid (GlycinePAS)
GSR-PAS	Granular slow release para-aminosalicylic acid
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HQC	High level quality control

INH	Isoniazid
IQR	Interquartile range
IS	Internal standard
k	Selectivity
KMN	Kanamycin
LC	Liquid chromatography
LC-MS	Liquid chromatography Mass spectrometry
LEV	Levofloxacin
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LNZ	Linezolid
LOD	Limit of detection
M Tuberculosis	Mycobacterium tuberculosis
m/z	Mass to charge ratio
MA	Massachusetts
MAP	*m*-Aminophenol
MBC	Minimum bactericidal concentration
MDR-TB	Multi drug resistant tuberculosis
MIC	Minimum inhibitory concentration
MO	Missouri
MOR	Morphazinamide
MQC	Medium level quality control
MRM	Multiple reaction monitoring
MS	Mass spectrometer / spectrometry
MTBE	Methyl tert-butyl ether
MXF	Moxifloxacin

N	Chromatographic efficiency (plate count)
N-5-ASA	*N*-acetyl-5-aminosalicylic acid
NAT	*N*-acetyltransferases
NAT1	Monomorphic *N*-acetyltransferase
NAT2	Polymorphic *N*-acetyltransferase
NCA	Non-compartmental analysis
NJ	New Jersey
NP-LC	Normal phase liquid chromatography
NSAIDS	Nonsteroidal anti-inflammatory drugs
P	Statistical Significance
PABA	*para*-Amino benzoic acid
PAS	*para*-Aminosalicylic acid
PD	Pharmacodynamics
pH	Potential hydrogen
PK	Pharmacokinetics
PPE	Protein precipitation experiments
PPI	Proton pump inhibitors
PPT	Protein precipitation
PTM	Prothionamide
PZA	Pyrazinamide
QCs	Quality control samples
RFM	Rifampicin
RP-LC	Reverse phase liquid chromatography
R_s	Retention factor
RSD	Relative standard deviation
RTM	Roxithromycin

SD	Standard deviation
SPE	Solid phase extraction
STP	Streptomycin
$t_{1/2}$	Half-life
TB	Tuberculosis
TDM	Toxicological and therapeutic drug monitoring
THF	Tetrahydrofolate
THIA	Thiacetazone
thyA	Thymidylate synthase
T_{max}	Time is reach maximum concentration
UHPLC	Ultra-high performance liquid chromatography
USA	United States of America
UV	Ultraviolet
VAS	Visual analogue scale
V_d	Volume of distribution
WHO	World Health Organisation
XDR-TB	Extensively drug resistant tuberculosis
α	Column efficiency

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Introduction and Outline

Tuberculosis (TB), despite its decline in incidence rates, remains the leading cause of death worldwide from a single infectious disease.[1] This global epidemic remains a public health concern in sub-Saharan Africa, due to the prevalent co-infection with the human immunodeficiency virus (HIV) and the occurrence of drug resistant TB (DR-TB).[1–4]

Compared to drug susceptible TB (DS-TB), multi-drug resistant (MDR) and extensively drug-resistant (XDR) TB is difficult to treat as it requires longer course duration and the use of chemotherapeutics with a higher toxicity profile.[5,6] Studies have found that relatively little advancement has been made in XDR-TB treatment since one of its first reports in South Africa, 10 years ago.[7,8]

Among the drugs used to treat DR-TB is *para*-aminosalicylic acid (PAS), one of the first anti-bacterial drugs discovered.[9] At the time of its inception, PAS was used as a core anti-TB drug, until drugs with increased efficacy and improved intolerance became available.[10,11] Due to the lack of use of PAS for many decades, it has acquired low resistance.[11] Presently, the WHO[1,12] has recommended use of PAS as a group C medicine, in the management of MDR- and XDR-TB regimens, to strengthen the regimen or as an alternative when priority drugs cannot be used.[12] The primary barrier of PAS use is its widely documented toxic profile. Occurrence of severe adverse effects related to PAS is reported to be one of the highest amongst drugs used in DR regimens.[8] Mostly reported are gastrointestinal distress, such as nausea, vomiting and diarrhoea.[13–15] Identifying the probable causes of these adverse reactions associated with PAS, would assist in managing its toxicity and improving its use. This presented study explored pharmacokinetic parameters of PAS and its main metabolites to identify co-variates that may play a role in gastrointestinal toxicity, such as the concentrations at which metabolites and parent drug are present in the plasma.

While newer drugs bedaquiline and delaminid are apparently better tolerated, treatment failures have already been reported as a result of newly emerged drug resistance.[16] Consequently, XDR-TB treatment failures are pooling in patients who remain therapeutically destitute. Under these circumstances, it is worthy to consider revision and repurposing of older drugs, such as PAS.

Discussed below is a general outline of the work presented in each chapter;

In **Chapter One**, a general introduction and rational is presented in order to state the purpose and value of the proposed study. The chapter discusses the epidemiology, current available treatment strategies of uncomplicated TB and DR-TB. The discussion continues regarding with the basic pharmacokinetics (PK) and pharmacodynamics (PD) characteristics of anti-TB drugs. Thereafter, a literature review of the anti-TB agent, para-aminosalicylic (PAS) and its metabolites Acetyl-PAS and Glycine-PAS is presented, highlighting its pharmacokinetics, mode of action, current dosing regimen and toxicological adverse effects.

Chapter Two is an outline of the analytical techniques employed. A large component of the study was the development and validation of a method to assay PAS and its metabolites using the currently available technology, namely liquid chromatography (LC) coupled to a mass spectrometry (MS) detector. An overview of the appropriate LCMS instrumentation used and the fundamentals of method development is discussed. The chapter also presents a literature review of the analytical methods previously reported for the determination of PAS and its metabolites since its introduction.

The study aims and objectives are described shortly in **Chapter Three**.

The study research question is addressed in **Chapter Four**. The experimental techniques that have been used are described which includes LCMS method development and validation,

non-compartmental analysis (NCA) for the PK evaluation of each compound in question, and the various statistical software used to summarise collected data and determine the relationship between concentration of each compound and the experienced adverse effect. Characteristics of the population in this study are described, as well as results, discussion and conclusions.

In **Chapter Five**, a discussion of the results generated is presented, and followed by conclusions, limitations and future work.

This thesis also includes the method development and validation of esomeprazole and its three major metabolites, which is described in **Addendum A**. Although this chapter's work is unrelated to the core study, it has been included to show the skill sets developed throughout this degree. The chapter presents a novel sample extraction method used to assay esomeprazole and its three major metabolites for the purpose of a pharmacokinetic study, which has been published and of which I am a co-author. It has been included as **Addendum B**: Cluver CA, Hannan NJ, van Papendorp E, Hiscock R, Beard S, Mol BW, Theron GB, Hall DR, Decloedt EH, Stander M, Adams KT, Rensburg M, Schubert P, Walker SP, Tong S. Esomeprazole to treat women with preterm preeclampsia: a randomized placebo controlled trial. Am J Obstet Gynecol. 2018 Jul 25. Pii: S0002-9378(18)30606-9. doi: 10.1016/j.ajog.2018.07.019. [Epub ahead of print] PubMed PMID: 30055127.

References

1. World Health Organization. Global tuberculosis control. WHO report. Geneva: World Health Orgnization. 2017. (WHO/HTM/TB/2017).

2. Dheda K, Migliori GB. The global rise of extensively drug-resistant tuberculosis: is the time to bring back sanatoria now overdue? Lancet. 2012; 379(9817):773–775.

3. Dheda K, Shean K, Zumla A, Badri M, Streicher EM, Page-Shipp L, et al. Early treatment outcomes and HIV status of patients with extensively drug-resistant tuberculosis in South Africa: a retrospective cohort study. Lancet. 2010; 375(9728):1798–1807.

4. Dheda K, Warren RM, Zumla A, Grobusch MP. Extensively drug-resistant tuberculosis: epidemiology and management challenges. Infect Dis Clin. 2010; 24(3):705–725.

5. Pooran A, Pieterson E, Davids M, Theron G, Dheda K. What is the Cost of Diagnosis and Management of Drug Resistant Tuberculosis in South Africa? PLoS One. 2013; 8(1). e54587. doi:10.1371/journal.pone.0054587.

6. Tiberi S, Scardigli A, Centis R, D'Ambrosio L, Muñoz-Torrico M, Salazar-Lezama MÁ, et al. Classifying new anti-tuberculosis drugs: rationale and future perspectives. Int J Infect Dis. 2017; 56:181–184.

7. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, et al. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet. 2006; 368(9547):1575–1580.

8. Bastos ML, Lan Z, Menzies D. An updated systematic review and meta-analysis for treatment of multidrug-resistant tuberculosis. Eur Respir J 2017; 49(3).

9. Donald PR, Schaaf HS. Old and new drugs for the treatment of tuberculosis in children. Paediatr Respir Rev. 2007; 8(2):134–141.

10. Iseman MD. Tuberculosis therapy: past, present and future. Eur Respir J. 2002; 20(36):87-94.

11. Momekov G, Momekova D, Stavrakov G. Para-Aminosalicylic Acid–Biopharmaceutical, Pharmaco-Logical, and Clinical Features and Resurgence As an

Anti-. BsphsOrg. 2015; (March).

12. World Health Organization. Rapid Communication : Key changes to treatment of multidrug- and rifampicin-resistant tuberculosis (MDR/RR-TB). WHO 2018; (August).

13. Lehmann J. The treatment of tuberculosis in Sweden with para-aminosalicylic acid; a review. Dis Chest. 1949; 16(6):684–703.

14. Carstensen B. Para-aminosalicylic acid (PAS) in pulmonary and extrapulmonary tuberculosis. Am Rev Tuberc Pulm Dis. 1950 ;61(5):613–620.

15. Budha NR, Lee RE, Meibohm B. Biopharmaceutics , Pharmacokinetics and Pharmacodynamics of Antituberculosis Drugs. Curr Med Chem. 2008; 15:809–825.

16. Bloemberg G V, Keller PM, Stucki D, Trauner A, Borrell S, Latshang T, et al. Acquired resistance to bedaquiline and delamanid in therapy for tuberculosis. N Engl J Med. 2015; 373(20):1986–1988.

Chapter One

Literature review – Antituberculosis agent, *para*-aminosalicylic acid (PAS)

1.1. Epidemiology of tuberculosis

Tuberculosis (TB) is a chronic infectious disease caused by the acid-fast bacillus *Mycobacterium tuberculosis* (*M. tuberculosis*).[1] It typically affects the lungs (pulmonary TB), but can also affect other sites (extrapulmonary TB). In 2016, there were an estimated 10.4 million (range 8.8 million – 12.2 million) incident cases of TB globally, with 1.30 million deaths (range, 1.20 million – 1.40 million) among individuals not infected with the human immunodeficiency virus (HIV) and an additional 0.37 million deaths (0.35 million – 0.43 million) among HIV-infected individuals (**Figure 1.1**). While the global TB incidence is falling by 2% per annum, it remains the ninth leading cause of mortality worldwide, and the leading cause from a single infectious agent.[2] It is estimated that Nigeria and South Africa each accounted for 4% of the total global number of cases as of 2016.

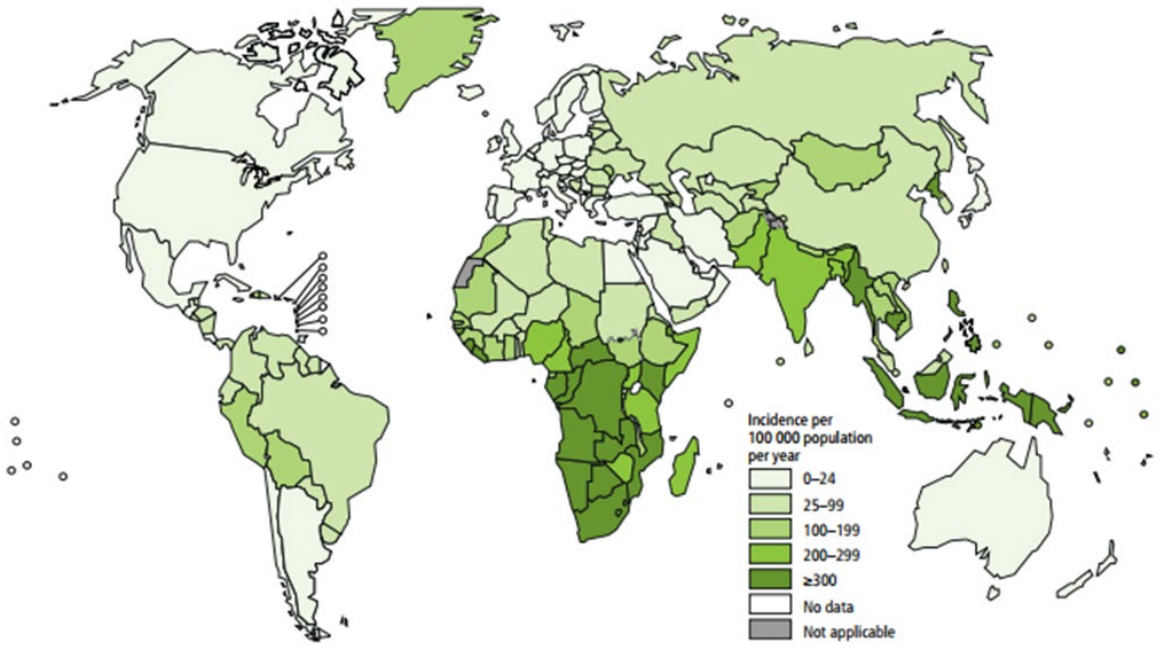

Figure 1.1: Global estimated TB incidence rate in 2016, for incidence per 100 000 population per year. [2]

With the use of modern molecular genetic techniques, Gutierrez and colleagues estimate an early progenitor present in East Africa as early as 3 million years ago. It is believed that early hominids were infected at this time.[3] Robert Koch, a German physician, announced his discovery and identification of the tubercle bacillus in 1882.[4] During this time, the only available treatment consisted of rest, a rich diet with some alcohol and carefully supervised exercise and resulted in the opening of sanatoria, which were facilities devoted to this anti-TB regimen. This approach proved more successful in healing patients than any previous treatment regimens.[5]

The discovery of streptomycin in 1944 was the first advancement of treating TB pharmacologically. This was followed by the introduction of isoniazid in the 1950's, which shaped the primary basis of anti-TB chemotherapy until the 1960's. During this time, the general anti-TB drug regimen consisted of streptomycin, isoniazid and para-aminosalicylic acid (PAS) in the initial months of treatment. The subsequent months included isoniazid and PAS, requiring a total treatment duration of 18 months. The discovery of rifampicin in 1965 prompted the launch of a short course treatment for TB in 1970. The directly observed treatment, short course (DOTS) strategy, when properly implemented with the necessary infrastructure, produces high TB cure rates and curtails the development of acquired drug-resistant TB.[6]

According to the South African National Tuberculosis Management Guidelines 2014 (**Table 1.1**), the current standard first-line treatment constitutes an intensive (initial) phase of two months consisting of rifampicin, isoniazid, pyrazinamide, and ethambutol, to rapidly kill the

mycobacteria. To prevent subsequent relapse, a continuation phase consisting of rifampicin and isoniazid is followed for a period of four months.[7,8]

Table 1.1: Standard treatment regimen of core first line drugs for known drug susceptible TB. [8]

Pre-treatment body weight (kg)	Intensive Phase (7 days a week for 2 months)	Continuation Phase (7 days a week for 4 months)	
	RHZE (150; 75; 400; 275) mg	RH (150; 75) mg	RH (300; 150) mg
30-37	2 tablets	2 tablets	
38-54	3 tablets	3 tablets	
55-70	4 tablets		2 tablets
>70 kg	5 tablets		2 tablets

R = rifampicin; H = isoniazid; Z = pyrazinamide; E = ethambutol; pyridoxine (Vitamin B6) 25 mg daily recommended for patients to prevent neuropathy commonly caused by isoniazid.

1.2. Multidrug- and Extensively drug-resistant tuberculosis

Multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB), is defined as strains of *M. tuberculosis* that are resistant to at least isoniazid and rifampicin, two of the most effective first line drugs used in the treatment of drug susceptible TB.[1] In the case of XDR-TB, the strains have an additional resistance to one of the fluoroquinolones, as well as one of the second line injectable drugs such as amikacin, kanamycin or capreomycin.[9] The surfacing of drug resistance in conjunction to co-infection with HIV, threatens global TB care and prevention, and remains a major public health concern in several countries.

It is estimated that globally 600 000 (range, 540 000 - 660 000) incident cases were attributed to MDR-TB, which constitutes 22% of the global TB burden. Furthermore, South Africa contributes approximately 1-2% to this MDR-TB occurrence.[2] A review by Andrews *et al.*[9] described the discovery of large numbers of cases of MDR-TB and XDR-TB in South Africa, as an evolving epidemic rather than sporadic localized outbreaks. It was noted that the prevalence of MDR-TB remained low in the mid-1990s but soared in the latter half of the decade.[10–12] Between 1993 and 1997 twenty-one individuals in Cape Town acquired TB resistance to four core drugs – isoniazid, rifampicin, ethambutol, and streptomycin – and were found to be HIV negative. These findings indicated that TB drug resistance was not necessarily associated with HIV co-infection.[13,14] In 2006 primary drug resistance was found associated with recent transmission and due to nosocomial transmission in an outbreak of MDR-TB and XDR-TB at the Church of Scotland Hospital in rural Tugela Ferry, KwaZulu-Natal. Systemic surveillance conducted at the hospital between January 2005 and March 2006 discovered that amongst 542 patients with positive sputum TB cultures, 221 (41%) had MDR-TB and 53 (9.7%) had XDR-TB, respectively.[15] The mortality of XDR-TB patients, which included several health care workers, was as high as 98%. The majority of patients with XDR-TB had no prior TB or HIV infections, thus indicating the cause of this outbreak to be inadequate infection control.[9,15,16]

Standard treatment regimens have proven inadequate for patients with drug resistance TB. Patients with XDR-TB are especially difficult to treat, and have a significantly worse treatment outcome than MDR-TB cases. As described in drug susceptible TB, a combination treatment plan is imperative to prevent additional resistance. Treatment possibilities include an individualised regimen based on results achieved from drug-susceptibility testing (DST), which is usually used in MDR-TB patients with a history of using second-line drugs.[8]

Typically, in patients with resistance to the standard regimen (rifampicin, isoniazid, pyrazinamide, ethambutol) a treatment plan including aminoglycosides (amikacin, kanamycin, capreomycin), a fluoroquinolone (levofloxacin, moxifloxacin), and an oral bacteriostatic agent (para-aminosalicylic acid, cycloserine, ethionamide, prothionamide) is used among other drugs.[17] It is crucial that individualised treatment is designed for patients with XDR-TB, in order to establish a suitable regimen.[8]

The recommended duration of treatment is guided by culture conversion and is determined by adding 18 months to the culture conversion dates. A minimum of five drugs should be included in the intensive phase, and four drugs in continuation phase as per the standard regimen (**Table 1.2**).

Table 1.2: Standardised MDR-TB regimen for adults (including children 8 years and older).[8]

Intensive Phase: 6 months or minimum of 4 months				
Drug	**Body weight (kg)**			
	< 33 kg	**33 – 50 kg**	**51 – 70 kg**	**> 70 kg**
Kanamycin	15 – 20 mg/kg	500 – 750 mg	1000 mg	1000 mg
Moxifloxacin	400 mg (children: 7.5 – 10 mg/kg)	400 mg	400 mg	400 mg
Ethionamide	15 – 20 mg/kg	500 mg	750 mg	750 – 1000 mg
Terizidone	5 – 20 mg/kg	750 mg	750 mg	750 – 1000 mg
Pyrazinamide	30 – 40 mg/kg	1000 – 1750 mg	1750 – 2000 mg	2000 – 2500 mg
Or, in children < 8 years				
Amikacin	15 – 22.5 mg			
Levofloxacin	10 – 15 mg			
Or, if known inhA mutation				
Ethambutol	20 – 25 mg			
High dose Isoniazid	15 – 20 mg			
Continuation Phase: Minimum of 18 months after TB culture conversion				
Drug	**Body weight (kg)**			
	< 33 kg	**33 – 50 kg**	**51 – 70 kg**	**> 70 kg**
Moxifloxacin	400 mg	400 mg	400 mg	400 mg
Ethionamide	15 -20 mg	500 mg	750 mg	750 – 1000 mg
Terizidone	15 – 20 mg	750 mg	750 mg	750 – 1000 mg
Pyrazinamide	30 - 40 mg	1000 – 1750 mg	1750 – 2000 mg	2000 – 2500 mg
Or, intolerance to moxifloxacin				
Levofloxacin	750 mg (below 51 kg)	1000 mg (equal or above 51 kg)		

InhA mutation = low level resistance to isoniazid. Pyridoxine (Vitamin B6) 150 mg to be given daily to patients on terizidone or cycloserine.

Recently, the WHO reviewed new evidence received individually from clinical trials, cohort/observational studies and programmatic implementation of both longer and shorter MDR-TB regimens. A rapid communication was published which highlights the new standard of care for MDR-TB treatment, based on evidence for effectiveness and safety.[18] The regimen consists of a revised grouping of TB drugs recommended for use in longer MDR-TB regimens (18 – 20 months), and is designed to include at least five drugs considered to be efficacious (**Table 1.3**). The use of injectables, capreomycin and kanamycin, is no longer recommended given their increased risk for adverse reactions and treatment failure when they are used in longer MDR-TB regimens.

Table 1.3: Revised grouping of drugs recommended for use in longer MDR-TB regimens.[18]

Group	**Drug**
Group A:	
Drugs to be prioritised (Include all three drugs, unless they cannot be used)	Levofloxacin/Moxifloxacin, Bedaquiline, Linezolid
Group B:	
Drugs to be added next (Add both drugs, unless they cannot be used)	Clofazamine, Cycloserine/Terizidone
Group C:	
Drugs to be included to complete regimens (Add when agents from group A and group B cannot be used)	Ethambutol, Delamanid, Pyrazinamide, Imipenem-cilastatin/Meropenem, Amikacin/Streptomycin, Ethionamide/Prothionamide/*p*-aminosalicylic acid

1.3. Anti-tuberculosis agents

The main desired properties of an anti-TB agent is to rapidly eliminate actively metabolizing bacilli, sterilize TB lesions and prevent resistance to companion drugs.[19,20] These three activities are determined by the dynamic combination of host, microbe and drug factors.[20] In pharmacology, this relationship is described by the principles of pharmacokinetics and pharmacodynamics.

1.4. Pharmacokinetics

Pharmacokinetics (PK) describes the time course of absorption, distribution, metabolism and excretion (ADME) of a drug in bodily fluids.[19,21] Essentially, the process of ADME characterizes the rise and fall of drug concentrations over time, known as the drug's pharmacokinetic profile.[22] Using this profile, mathematical models can be applied to determine the pharmacokinetic parameters of the drug. Parameters such as maximum concentration (C_{max}), minimum concentration (C_{min}), time to reach the maximum concentration (T_{max}), area under the curve (AUC) bioavailability (F), clearance (CL), half-life ($t_{1/2}$) and volume of distribution (V_d) can be quantified and used to determine optimal drug dosage regimens.[23]

1.5. Pharmacodynamics

Pharmacodynamics (PD) is defined as the drug's effect in relation to its concentration in the body.[19] Generally, PD refers to what the drug does to the body, however, antimicrobial PD

describes the relationship between the drug concentration and its effect on the pathogen residing within the host organism.[19,21] Based on the antimicrobial pharmacodynamic action of a drug, it can be classified as bacteriostatic or bactericidal.[24]

Bacteriostatic agents inhibit the growth of bacilli, keeping them in the stationary phase of growth and relying on the host's defence mechanism to remove bacilli from the tissues.[24,25] Should the host defences fail to do so, the bacilli will resume growth after discontinuation of the bacteriostatic agent. Currently TB is treated with combination regimens consisting of bacteriostatic and bactericidal agents.[26] The latter group, rapidly reduces the size of the bacillary population in the sputum of TB patients after starting the treatment, and prevents the emergence of resistance to companion drugs.[27]

PD parameters quantify the activity of an antimicrobial agent pertaining to growth inhibition and eradication of the pathogen[22] and this activity can be determined *in vitro* or *in vivo*. The minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) are the major parameters, which quantify the potency of the antimicrobial agent *in vitro*. The MIC represents the lowest drug concentration that inhibits microbial growth after incubation for 16-20 hours with a standard inoculum of 1-5 x 10^5 colony forming units (CFU/mL).[19,28] The MBC refers to the drug concentration that kills 99.99% of the initial inoculum. By contrast, *in vivo* activity is described by the early bactericidal activity (EBA) of a drug, which measures the decreasing rate of CFU/mL in the sputum of patients during the first two days of drug treatment and is expressed as $\log_{10}$ (CFU/mL)/day.[19]

In order to actively inhibit the infectious pathogen, the concentration of the bacteriostatic agent must remain above the MIC of the pathogen. Bactericidal activity can follow two

patterns, either time-dependent activity or concentration-dependent activity. In time-dependent activity, killing of the bacteria depends on the duration of drug exposure, whereas concentration-dependent killing requires the maximum concentration possible at the site of infection.[27,29] The Cmax to MIC or AUC to MIC ratios correlate best with the eradication rates, as demonstrated in **Figure 1.2**.

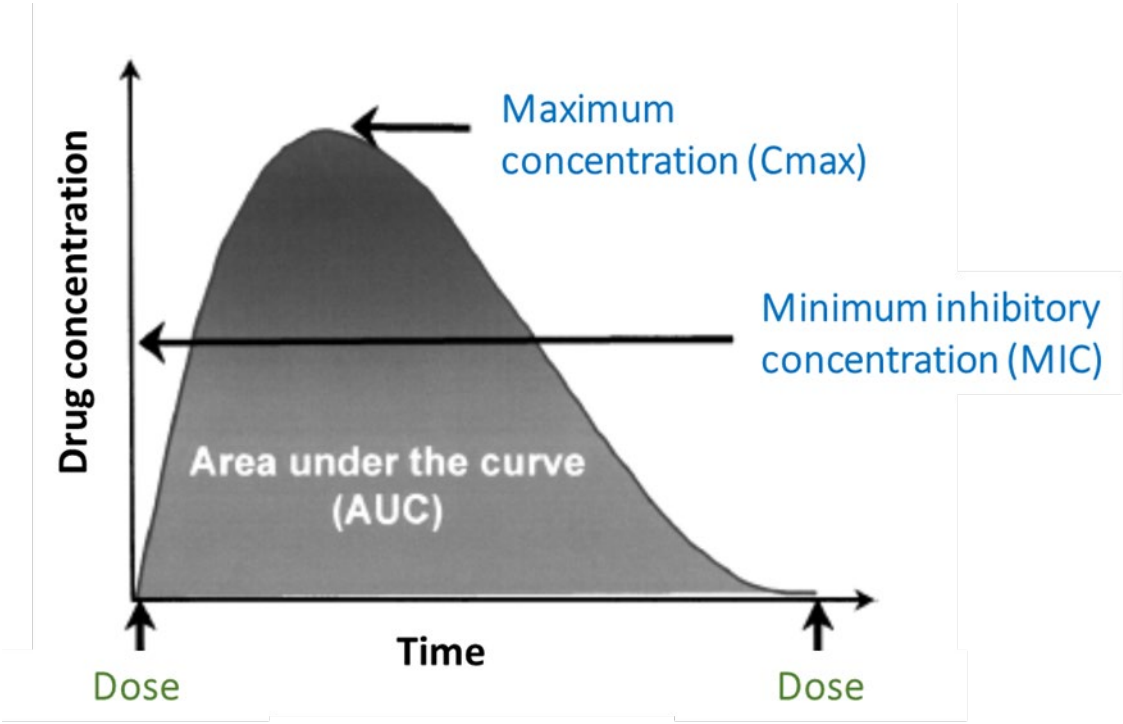

Figure 1.2: AUC/MIC and Cmax/MIC ratios, correlation of serum pharmacokinetics (Cmax, AUC) with MIC (susceptibility) of an organism, which best describes eradication rate. The MIC at which the magnitudes of these ratios that are required for clinical success are achieved becomes the pharmacokinetic/pharmacodynamic profile of the drug. Adapted from Jacobs[29]

1.6. Factors influencing PK/PD profile of anti-TB agents

Inter subject variability in drug response has been a major problem in drug development and clinical practice as a whole. As a consequence, it can lead to the risk of developing an adverse drug reaction or therapeutic failure. Potential factors responsible for subject

variability in drug response include the patient's age, disease state (particularly liver and renal function), concomitant drug therapy, genetic variability and lifestyle behaviour such as smoking and alcohol consumption.[30] Of even greater importance in the therapeutic and toxic profile of a drug is the biotransformation process, which is responsible for detoxification, excretion and/or activation, by drug metabolizing enzymes.[31] Generally biotransformation pathways include the oxidation, hydrolysis or reduction of the parent drug (phase I enzyme), followed by conjugation of the oxidised moiety with highly polar molecules such as sulphate and glucuronic acid (phase II enzyme). Biotransformation of a drug can be mediated by phase I, phase II or a combination of both pathways.[31,32] Phase I consist primarily of the cytochrome P450 (CYP) superfamily of enzymes, which are abundant in the liver, gastrointestinal tract, the lungs and kidneys.[33] Among the phase II conjugating enzymes are *N*-acetyltransferases (*NAT*), which are present in various isoforms that are substrate specific. Typically, conjugation reactions enhance the excretion of a drug by increasing its hydrophilicity, and thereby exerting a detoxification effect. However, under certain conditions, conjugation reactions may result in activated metabolites and increased toxicity by xenobiotics.[32] As mentioned above, subject variability largely contributes to altered drug metabolism, as a result of factors including genetic variation of drug metabolizing enzymes, the presence or absence of diseases such as TB and HIV and the use of concomitant medications.[30]

1.7. Intolerance of anti-TB agents

Studies have shown that the occurrence of adverse drug reactions contributes significantly to morbidity and patient hospitalization.[34,35] The frequency, severity and nature of anti-TB drug induced adverse reactions have always been a matter of concern. Therefore, the clinical

monitoring of all TB patients during treatment is of importance, as serious adverse reactions induced by anti-TB therapy may lead to non-compliance of patients and consequently, treatment failure or emergence of drug resistant *M. tuberculosis*. First line anti-TB agents may cause gastrointestinal disturbance and hepatotoxicity.[8,36] Anti-TB agents used in MDR- and XDR-TB regimens are associated with more and worse adverse effects, and therefore create a big challenge in the management and treatment of patients with drug resistant TB.[37]

1.8. Para-aminosalicylic acid

Para-aminosalicylic acid (PAS, *p*-aminosalicylic acid, 4-aminosalicylic acid, 4-ASA) was one of the first chemotherapeutic agents found to be effective in the treatment of *M. tuberculosis*.[38] Before more potent drugs became available, PAS was used in combination therapy and showed its substantial efficacy and ability to prevent resistance in its companion drugs, allowing it to be part of standard TB treatment.[39] However, shortly after its discovery, PAS was noticed to cause severe gastrointestinal (GI) intolerance, resulting in its replacement with better tolerated and equally efficacious drugs, such as ethambutol and isoniazid.[40] Amid the widespread HIV epidemic and rates of resistance to first-line and second-line drugs, PAS usage has gained a new life.[41]

The manufacturing of a new granular slow release PAS (GSR-PAS) formulation with fewer GI adverse effects (AEs)[42–44] has supported its reintroduction. Another benefit is the low drug resistance to PAS since its use was uncommon for several decades.[42] It is now primarily used as a second-line drug to strengthen regimens for patients who have XDR-TB, or have not responded to standard MDR-TB regimens.[41] It has been implemented in the

current WHO Guidelines for the programmed management of DR-TB.[17,26] Presently, the GSR-PAS formulation is used in the USA, Europe and many other countries.[41,44,45]

1.8.1. Mechanism of action of PAS

PAS is classified as a highly specific bacteriostatic agent, inhibiting the growth of *M. tuberculosis* at a concentration of 1 µg/mL *in vitro*.[19] Even though PAS is widely researched and used in clinical practice, its exact mechanism of action remains elusive, but three mechanisms have been considered.

Firstly, it is speculated that PAS inhibits folic acid synthesis, by acting as a competitive inhibitor of *para*-amino benzoic acid (PABA).[46] Structural similarities between PAS and sulphonamides, which are well studied analogues of PABA, led to the suggestion that PAS might compete with PABA for dihydropteroate synthase (DHPS), a key enzyme in the folate biosynthesis pathway of bacteria (**Figure 1.3**).[42]

Secondly, in contrast to sulphonamides, PAS appears to be a poor inhibitor of DHPS *in vitro*, suggesting the possibility for an alternative target.[47] Rengarajan *et al*.[42] identified clinical PAS-resistant isolates of *M. tuberculosis* that had mutations in the thymidylate synthase (*thyA*) gene, which resulted in decreased enzyme activity. As *thyA* modulates the enzyme needed to determine intracellular folate levels, it is suggested that PAS acts as a folate antagonist, which inhibits folic acid synthesis (**Figure 1.3**).

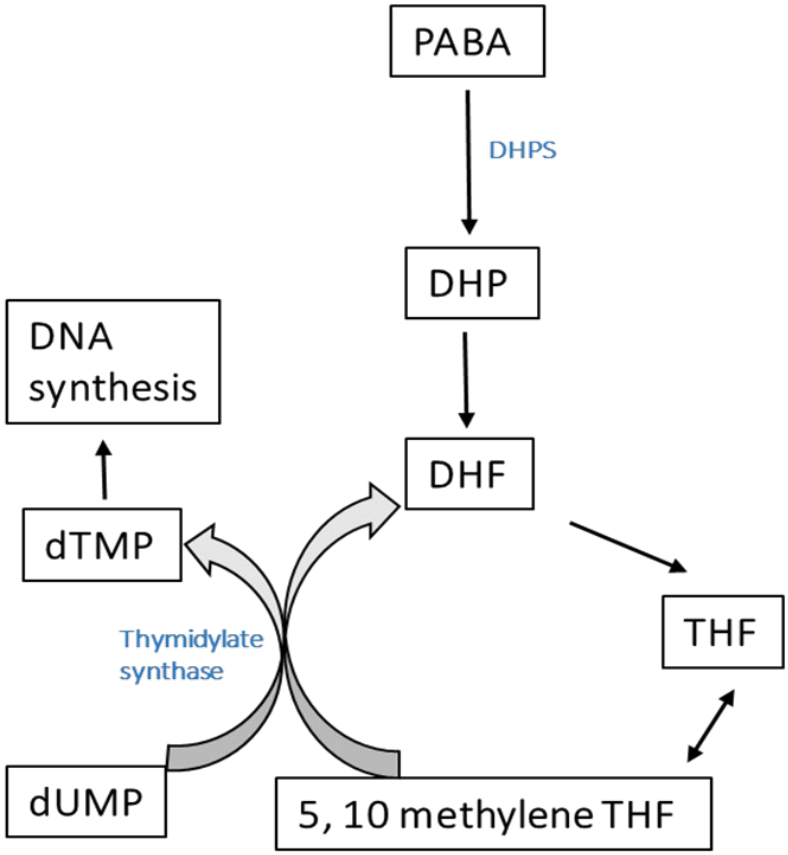

Figure 1.3: Postulated mechanism of action of PAS in the folic acid biosynthesis pathway. PABA, para-aminobenzoic acid; DHPS, dihydropteroate synthase; DHP, dihydropteroate; DHF, dihydropfolate; THF, tetrahydrofolate; dUMP, deoxyuridine 5'-monophosphate; dTMP, deoxythymidine 5'-monophosphate. Adapted from Rengarajan.[42]

Thirdly, another suggested the mechanism of action, is the ability of PAS to inhibit the synthesis of mycobactins, a cell wall component that is crucial for the iron transport in *M. tuberculosis*, thereby preventing the bacilli from acquiring iron from the host and reducing the activity of bacterial enzymes that require iron.[48–50]

1.8.2. Pharmacokinetics of PAS

After PAS was introduced, many formulations were manufactured, including various salts, enteric coated granules, a PAS-resin complex and later encapsulated gastro-resistant granules, to prevent early breakdown in the stomach.[41] The drug's bioavailability largely relies on the chemical species and the dosage form administered (**Table 1.4**).[26,51]

Table 1.4: Summary of pharmacokinetic characteristics of different formulations of PAS, administered orally in healthy subjects. Adapted from Berning[52] and Momekov[26].

PAS preparation	Dose (g)	C_{max} (µg/mL)	T_{max} (h)	$AUC_{0-\infty}$ (µg/h/mL)
Free PAS	4.0	49.98	3.54	209.07
Sodium PAS	2.8	155.44	0.83	313.22
Calcium PAS	2.6	139.51	1.02	326.22
Potassium PAS	2.6	121.09	1.10	313.22
PAS resin complex	4.0	78.0	2.1	-
GSR-PAS	4.0	20.23	7.95	107.92

GSR-PAS = granular slow release formulation.[44]

PAS is completely absorbed from the gastrointestinal tract (GIT) after oral administration, with peak plasma concentrations occurring within 2 hours after ingestion. Typically, in the slow release formulation, mean plasma maximum concentrations (C_{max}) of 20 – 60 µg/mL are achieved in 4 – 6 hours after a single 4g dose.[19,26,40] In contrast, among individuals with

MDR/XDR-TB, a mean C_{max} of 51.3 µg/mL was reached at a T_{max} (time to maximum concentration) of 5.2 hours.[43]

PAS is 50 – 60% bound to plasma proteins, and well distributed throughout the total body water. The highest concentrations are detected in the kidney, lungs, liver and to some degree in the cerebrospinal fluid (CSF), if the meninges are inflamed.[19,26] Following a conventional oral dose, PAS has a half-life of approximately1 hour, and minimal plasma concentrations reached within 4 – 5 hours.[51]

After 24 hours approximately 80% of the dose is excreted in the urine, by glomerular filtration and tubular secretion, with more than 50% in the metabolised form.[52,53] Elimination is affected by severe renal and/or hepatic dysfunction, and the use of PAS is therefore not recommended in such individuals.[26]

1.8.2.1. Metabolism of PAS

The metabolic breakdown of PAS occurs rapidly starting in the intestinal mucosa and continuing in the liver. The primary biotransformation is via acetylation of the free amino group.[54] This reaction forms *N*-acetyl-para-aminosalicylic acid (APAS, acetyl-PAS) and is mediated by *N*-acetyltransferase 1 (*NAT1*).[19,51] In addition to acetylation there is also glycine conjugation of the carboxyl group to form *p*-aminosalicyluric acid (GPAS, glycine-PAS).[51] Due to a limited number of drugs being conjugated with glycine, the pathway has not been characterised.[55] Although APAS and GPAS are the main products, other minor metabolites have been identified in the urine, including ether glucuronides of PAS (**Figure 1.4**).[56]

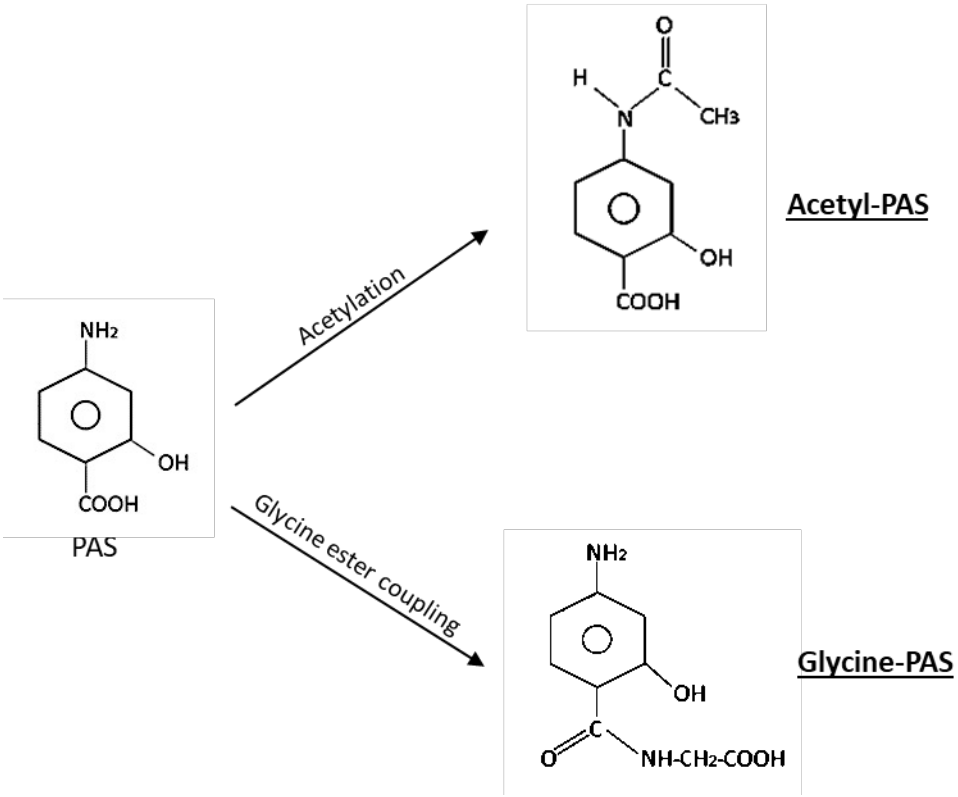

Figure 1.4: Conjugations reactions of PAS by acetylating enzymes producing Acetyl-PAS (Ac-PAS, APAS), by glucoronate and sulphate conjugating enzymes, and by glycin conjugating enzymes producing Glycyl-PAS (Glycine-PAS, Gly-PAS, GPAS). Adapted from Lehmann.[56]

When exploring the metabolic profile of PAS, it was noted that APAS is devoid of bacteriostatic activity against *M. tuberculosis*, with the exception of very high plasma concentrations, in the urine.[56] By contrast, GPAS exerts some anti-TB activity proportional to the dose of PAS.[56] The dose of PAS has also shown to have a significant effect on the acetylation and glycine-conjugation pathways as these processes are known to be saturable.[57] At a low dose (4g daily), PAS absorption is succumbed to rapid first-pass acetylation, thus causing APAS to be detected in the blood before unchanged PAS.[58] When a higher dose (8g daily) is administered, acetyl conjugation is saturated, resulting in significantly higher GPAS and free PAS plasma concentrations, which is believed to be due to the competition for a common substrate, coenzyme A, by the conjugating enzyme

systems.[56,57] Therefore, first pass effect greatly affects metabolism, and in turn the resulting plasma concentrations.[56]

1.8.2.2. Pharmacokinetic studies of PAS

Despite the many years that PAS has been used there is a paucity of pharmacokinetic data in the literature. A small number of studies have evaluated the pharmacokinetic characteristics of the metabolite APAS, and even fewer that of GPAS. Nevertheless, reports are available for studies conducted in healthy subjects and patients with drug susceptible and other patients with drug resistant TB, including adults and children with TB.[43,58–61]

In these studies, the recommended GSR-PAS formulation is evaluated, at different dosing strategies. Especially marked is the difference in release characteristics of the granules when compared to previous preparations. In adults receiving a single 4 g GSR-PAS, the mean C_{max} was around 20 µg/mL (SD; standard deviation = 8.80 µg/mL), 2 – 4 times less than the typical PAS tablets, and the T_{max} was 7.95 hours (SD = 6.12 hours) compared to 0.5 – 2 hours in the tablet. This allows PAS to be present longer and remain above the required MIC, in systemic circulation.[58,62,63] Serum concentrations of APAS was also reported, as mean C_{max} of 17.50 µg/mL (SD = 3.48 µg/mL) and T_{max} of 9.27 hours (SD = 5.24 hours). The majority of subjects had APAS detected in serum before PAS itself. These findings were consistent with previous reports of first pass metabolism.[56–58]

Inter-individual variation was significant in the pharmacokinetics of patients treated for MDR- and XDR-TB.[43,59] One study conducted in an adult South African population,

reported a mean C_{max}, T_{max} and $AUC_{0-\infty}$ of 51.3 µg/mL (SD = 20.0 µg/mL), 5.2 hours (SD = 2.04 hours) and 368.0 µg/h/mL (SD = 194.0 µg/h/mL) respectively, receiving a 4 g GSR-PAS dose. These findings were within the same range using the slow release formulation in a previously reported American population.[58] The latter study found that the coefficient of variation for C_{max} varied by 43.5% and T_{max} by 76.9%. The most probable reason for this may be individual capacity to absorb the PAS granules, which may result in overlapping absorption, distribution and elimination phases.[58]

In the evaluation of the effects of feeding on GSR-PAS absorption, changes in gastric pH produced by orange juice or antacids were not significant. However, absorption was improved by co-administration of a high-fat meal, which showed an increased C_{max} and delay in T_{max}, in turn increasing the time that concentrations remain above the target MIC.[60] Additionally, the potential effect of drug interactions among HIV-infected subjects concomitantly administered PAS and efavirenz (EFV), has been reported. Concurrent use of EFV showed approximately 52% increase in PAS clearance, and an overall reduction of 30% in the mean AUC.[61]

1.8.3. Dosing regimens of PAS

While it has been reported that up to 65% of patients with XDR-TB can be successfully treated, studies in South Africa found that within 2 years of diagnosis, as many as 50% and 80% of patients with MDR- and XDR-TB had died, respectively.[64–66] Despite its drawbacks, PAS is an essential drug in the treatment of patients with MDR-TB and especially patients with XDR-TB. A study conducted in South Africa identified three (6%) of 48 *M. tuberculosis* isolates from XDR-TB patients, demonstrating resistance to PAS.[65] Therefore, these circumstances call for a revision of the recommended use and dosing regimen of PAS.

The GSR-PAS preparation is locally available, and prescribed as 8 – 12 g in three divided doses.[7] In order to maintain plasma concentrations above the MIC of 1 µg/mL, a multiple dosing regimen is recommended, as with other typical bacteriostatic agents.[67] This regimen was derived from TB studies using earlier formulations, as the divided doses were thought to reduce the intolerance to PAS. [45,59] However, a number of earlier studies reported that once-daily dosing of PAS was better tolerated than multiple daily dosing.[56,68]

In the study by Marsden, which looked at different dosages and rhythm of administration of PAS it was reported that 119 (30%) of patients receiving divided sodium PAS doses experienced GI disturbances, compared to only 44 (12%) on a once-daily regimen.[69] Yue and Cohen[70] reported similar findings using the sodium salt preparation when 12 g PAS was given as 4 g three times daily (14.8%), 6 g twice-daily (15.5%), and 12 g once-daily (10.1%), respectively.[41] A Swedish study reported that PAS as a “single massive dose” was preferred by “about two thirds of the patients on PAS medication”.[71]

With the GSR-PAS formulation, significantly less intolerance has been observed with only a minority of patients experiencing vomiting, nausea, bloating, or diarrhoea. In a study by Peloquin[58] a single 4 g once-daily compared with 4 g twice-daily GSR-PAS dose was found to be equally well tolerated. In a similar investigation comparing a 4 g twice-daily and 8 g once-daily GSR-PAS dose, findings suggested that twice-daily dosing was associated with more intolerance than the once-daily dosing.[72] In addition, it was noted that abdominal pain and discomfort and diarrhoea occurred when minimum plasma concentrations of PAS were present. These findings led Sy and colleges[72] to the hypothesis that rapid absorption and biotransformation of PAS may potentiate GI intolerance, probably as a result of its metabolic entities being produced. However, their hypothesis could not be evaluated, as their study did not determine the metabolite concentrations.

1.8.4. Adverse effects of PAS

After the first use of PAS, its adverse effect profile was well characterized. Several formulations were manufactured to overcome the GI intolerance. These effects were reduced with the GSR-PAS, which allowed less production of meta-aminophenol, a potentially toxic degradation-product of PAS.[73] The slower release in the small intestines avoided gastric irritation, compared to the previously used salt preparations.[45]

The most commonly occurring AEs associated with PAS are hypothyroidism and GI intolerance, including nausea, bloating, vomiting, diarrhoea, abdominal pain and anorexia.[74] While mild GI effects were evident in up to 70% of patients using the older formulation, these are rarely severe enough to warrant discontinuation of the drug.[26]

Hypersensitivity reactions are seen in 5 – 10% of patients, manifesting as fever, joint pains, sore throat, and skin eruptions, which can occur independently or are accompanied by the fever. In a retrospective study of 7492 patients treated for TB, hepatitis developed in 38 (0.5%), from which 28 (0.3%) cases were attributed to the use of rapidly absorbed PAS preparations (i.e. salt and/or granules).[74] Symptoms such as jaundice appeared within 4 weeks after start of therapy. A high mortality rate for hepatotoxicity (21%) has been seen with continued use after symptoms are observed.[52]

Other rarely reported AEs include haematological aberrations, such as leukopenia, agranulocytosis, thrombocytopenia and acute haemolytic anaemia.[75] Hypokalemia, acidosis and crystalluria have occurred occasionally.[75,76]

A recent review of drug associated adverse effects with the use of PAS, reported that seven (7%) of 101 patients with XDR-TB had to stop taking PAS.[37] This figure is consistent with earlier studies, which reported intolerance in approximately 10% of patients.[77] GI adverse reactions were predominantly reported, including nausea, vomiting, diarrhoea and other gastrointestinal symptoms. The frequency of adverse effects were high (approximately 60%) in the studied XDR-TB treatment regimens, that included ethionamide, pyrazinamide, ethambutol and capreomycin.[37]

Due to the predominant use of PAS in DR-TB, the strategy of combined therapy is a concern, as the intolerance cannot be ascertained to PAS alone.[26] For this reason, the influence of drug interactions with PAS needs to be considered when determining the causative of the frequently observed GI adverse reactions.

1.8.5. Drug interactions with PAS

PAS is known to have interactions with other drugs, the most notable being the inhibiting effect on isoniazid acetylation during PAS therapy, causing isoniazid concentrations to be significantly higher when co-administered with PAS.[19,78] It has been reported that EFV increases the elimination of PAS, thereby potentially compromising therapeutic efficacy when administered together.[61] At present it is unclear whether significant interactions occur between PAS and other antiretroviral (ARV) drugs.

The identified pharmacokinetic and pharmacodynamic drug-drug interactions between PAS and other medicines are presented in **Table 1.5**.

Table 1.5: Reported pharmacokinetic/pharmacodynamic drug-drug interactions between PAS and other medicines.

Concomitant drug	Effect
Pharmacokinetic	
Digoxin	Decreases the absorption of PAS
Isoniazid	Inhibition of isoniazid acetylation leading to increased concentrations
Rifampicin	Absorption is reduced by PAS
Vitamin B12	PAS reduces serum concentrations of vitamin B12
Folate	Absorption is reduced by PAS
Probenecid	Increases serum concentrations of PAS

Adapted from Arbex[48], McIlleron[76], and Momekov[26].

Table 1.5: Reported pharmacokinetic/pharmacodynamic drug-drug interactions between PAS and other medicines (continued).

Concomitant drug	Effect
Pharmacodynamic	
Angiotensin converting enzyme inhibitors	PAS reduces the antihypertensive effects
Calcium channel blockers	Increases anticoagulant effect of PAS
Carbonic anhydrase inhibitors	Potentiate adverse effects of both drugs
Corticosteroids	Increases severity of corticosteroid adverse effects
Loop diuretics	PAS can reduce effects of loop diuretics, and the loop diuretics can increase serum concentrations of PAS
Non-selective NSAIDS (except diclofenac)	May increase adverse effects of PAS
Sulfonylurea	PAS increases hypoglycemic effects
Oral anticoagulants, thrombolytics, salicylates	Risk of bleeding is increased by PAS
Ethionamide	Potential increase in risk of hepatotoxicity and hypothyroidism
Methotrexate	PAS may increase toxicity of methotrexate
Azathioprine	Toxicity is increase by PAS
Tiaprofenic acid	Increases risk of gastrointestinal bleeding
Trandolapril	Efficacy may be reduced by PAS
Thioguanine	PAS may enhance toxicity
Warfarin	Antiplatelet effects of PAS may increase bleeding risk of warfarin
Tolmetin	Increases risk of gastrointestinal bleeding
Sulindac	PAS may decrease serum concentrations, and sulindac enhances risk of toxity such as bleeding
Azathioprine	Toxicity is increased by PAS
Treprostinil	Risk of bleeding is increased
Mercaptopurine	Toxic effects are increased by PAS

Adapted from Arbex[48], McIlleron[76], and Momekov[26].

1.8.6. Pharmacogenomics of PAS

Arylamine *N*-acetyltransferases are divided into two classes, polymorphic (*NAT2*) and traditionally designated monomorphic (*NAT1*).[79,80] Differences in the acetylating activity in isoniazid was the earliest identified genetic variation in drug metabolism. Substrates of *NAT2* include isoniazid, hydralazine and sulphamethazine, therefore inter-individual variation in N-acetylation is observed in these drugs. In contrast, monomorphic substrates such as PAS and PABA are metabolized by *NAT1*.[81,82]

Allelic variation of *NAT1* have been identified, which associate with decreased enzyme activity, although very rarely occurring.(83) As *NAT1* is chiefly responsible for metabolism of PAS, it has been used as a probe for *NAT1* function. Among the described polymorphisms the most common are *NAT1*10*, *NAT1*3* and *NAT1*11*, with reference to wild-type *NAT1*4*.[83,84]

A reduction in enzyme activity associated with the *NAT1*14* allele was found in a southern African population, consisting of African and mixed ancestry. No evidence was found to support increased activity associated with *NAT1*10*.[72,85] An interesting finding was particularly high PAS exposure in a patient with a *NAT1*10/1*3* genotype and two others carrying *NAT1*14* allele, implying reduced enzyme function.

The influence of *NAT2* genes on PAS metabolism is noted by Sy *et al*.[72] These findings reflect interactions between *NAT1* and *NAT2* genes, similarly to that reported in oncology studies,[86] which noted that *NAT1* and *NAT2* polymorphisms can interact with one another and increase or decrease the risk of cancer developments.[86]

References

1. Kvasnovsky CL, Cegielski JP, Erasmus R, Siwisa NO, Thomas K, der Walt ML van. Extensively Drug-Resistant TB in Eastern Cape, South Africa: High Mortality in HIV-Negative and HIV-Positive Patients. JAIDS J Acquir Immune Defic Syndr 2011; 57(2):146–152.

2. World Health Organization. Global Tuberculosis control. WHO. report. Geneva: World Health Organization. 2017. (WHO/HTM/TB/2017).

3. Cristina GM, Brisse S, Brosch R, Fabre M, Omaïs B, Marmiesse M, et al. Ancient origin and gene mosaicism of the progenitor of Mycobacterium tuberculosis. PLoS Pathog. 2005; 1(1):55–61.

4. Sakula A. Robert Koch: centenary of the discovery of the tubercle bacillus, 1882. Thorax. 1982; 37(4):246–251.

5. McCarthy OR. The key to the sanatoria. J R Soc Med. 2001 ;94(8):413–417.

6. Nuermberger EL, Spigelman MK, Yew WW. Current development and future prospects in chemotherapy of tuberculosis. Respirology. 2010; 15(5):764–778.

7. Department of Health Republic of South Africa. Management of Drug-resistant Tuberculosis policy guidelines 2013;1-162.

8. Department of Health Republic of South Africa. National Tubeculosis Management Guidelines. 2014.

9. Andrews JR, Shah NS, Gandhi N, Moll T, Friedland G. Multidrug-Resistant and Extensively Drug-Resistant Tuberculosis: Implications for the HIV Epidemic and Antiretroviral Therapy Rollout in South Africa. J Infect Dis. 2007; 196(s3):S482–S490.

10. Davies GR, Pillay M, Sturm a W, Wilkinson D. Emergence of multidrug-resistant tuberculosis in a community-based directly observed treatment programme in rural South Africa. Int J Tuberc Lung Dis. 1999; 3(9):799–804.

11. Weyer K, Groenewald P, Zwarenstein M, Lombard CJ. Tuberculosis Drug-resistance in the Western Cape. South African Med J. 1995; 85(6):499–504.

12. Wilkinson D, Pillay M, Davies GR, Sturm AW. Resistance to antituberculosis drugs in

rural South Africa: Rates, patterns, risks, and transmission dynamics. Trans R Soc Trop Med Hyg. 1996; 90(6):692–695.

13. Louw GE, Warren RM, Donald PR, Murray MB, Bosman M, Van Helden PD, et al. Frequency and implications of pyrazinamide resistance in managing previously treated tuberculosis patients. Int J Tuberc Lung Dis. 2006; 10(7):802–807.

14. Victor TC, Streicher EM, Kewley C, Jordaan AM, Van Der Spuy GD, Bosman M, et al. Spread of an emerging Mycobacterium tuberculosis drug-resistant strain in the Western Cape of South Africa. Int J Tuberc Lung Dis. 2007; 11(2):195–201.

15. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, et al. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet. 2006; 368(9547):1575–1580.

16. Cooke GS, Beaton RK, Lessells RJ, John L, Ashworth S, et al. Spread of MDR TB from Tugela Ferry, South Africa. Emerg. Infect. Dis. 2011;17(11):2035–2037.

17. Caminero JA, Sotgiu G, Zumla A, Migliori GB. Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis. Lancet Infect Dis. 2010; 10(9):621–629.

18. World Health Organization. Rapid Communication : Key changes to treatment of multidrug- and rifampicin-resistant tuberculosis (MDR/RR-TB). 2018; August.

19. Budha NR, Lee RE, Meibohm B. Biopharmaceutics , Pharmacokinetics and Pharmacodynamics of Antituberculosis Drugs. Curr Med Chem. 2008; 15:809–825.

20. Donald P. Antituberculosis drugs. 1st ed. Tuberculosis. Elsevier Inc.; 2009. 608-617.

21. Meibohm B, Derendorf H. Basic concepts of pharmacokinetic/pharmacodynamic (PK/PD) modeling. Int J Clin Pharmacol Ther. 1997; 35(10):401–413.

22. Burgess DS. Pharmacodynamic Principles of Antimicrobial Therapy in the Prevention of Resistance. Chest. 1999; 115(3):19S–23S.

23. Gunaratna C. Drug metabolism & pharmacokinetics in drug discovery: a primer for bioanalytical chemists, part I. Curr Sep. 2000; 19(1):17–24.

24. Pankey GA, Sabath LD. Clinical Relevance of Bacteriostatic versus Bactericidal Mechanisms of Action in the Treatment of Gram-Positive Bacterial Infections. Clin

Infect Dis. 2004 ;38(6):864–870.

25. Levison ME. Pharmacodynamics of antimicrobial drugs. Infect Dis Clin. 2004; 18(3):451–465.

26. Momekov G, Momekova D, Stavrakov G. Para-Aminosalicylic Acid–Biopharmaceutical, Pharmaco-Logical, and Clinical Features and Resurgence As an Anti-tuberculosis agent. Pharmacia. 2015; 62:25–51.

27. Nuermberger E, Grosset J. Pharmacokinetic and pharmacodynamic issues in the treatment of mycobacterial infections. Eur J Clin Microbiol Infect Dis. 2004; 23(4):243–255.

28. Waitz JA. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. National Committee for Clinical Laboratory Standards; 1990.

29. Jacobs MR. Optimisation of antimicrobial therapy using pharmacokinetic and pharmacodynamic parameters. Clin Microbiol Infect. 2001; 7(11):589–596.

30. Meyer UA. Adverse drug reactions Pharmacogenetics and adverse drug reactions. Lancet. 2000; 356:1667–1671.

31. Brandon EFA, Raap CD, Meijerman I, Beijnen JH, Schellens JHM. An update on in vitro test methods in human hepatic drug biotransformation research: Pros and cons. Toxicol Appl Pharmacol. 2003; 189(3):233–246.

32. Xu C, Li CY, Kong AN. Induction of phase I, II and III drug metabolism/transport by xenobiotics. ArchPharmRes. 2005; 28(3):249–268.

33. De Kock L. Pharmacokinetic study of twice-daily versus once-daily dosing with granular slow release para-aminosalicylic acid in adults on second-line anti-tuberculosis and antiretroviral treatment. MSc thesis, Stellenbosch University: 2013.

34. Yee D, Valiquette C, Pelletier M, Parisien I, Rocher I, Menzies D. Incidence of Serious Side Effects from First-Line Antituberculosis Drugs among Patients Treated for Active Tuberculosis. Am J Respir Crit Care Med. 2003; 167(11):1472–1477.

35. Mehta U, Durrheim DN, Blockman M, Kredo T, Gounden R, Barnes KI. Adverse drug reactions in adult medical inpatients in a South African hospital serving a community with a high HIV/AIDS prevalence: prospective observational study. Br J Clin

Pharmacol. 2008; 65(3):396–406.

36. Javadi MR, Shalviri G, Gholami K, Salamzadeh J, Maghooli G, Mirsaeedi SM. Adverse reactions of anti-tuberculosis drugs in hospitalized patients: incidence, severity and risk factors. Pharmacoepidemiol Drug Saf. 2007; 16(10):1104–1110.

37. Shean K, Streicher E, Pieterson E, Symons G, van Zyl Smit R, Theron G, et al. Drug-Associated Adverse Events and Their Relationship with Outcomes in Patients Receiving Treatment for Extensively Drug-Resistant Tuberculosis in South Africa. PLoS One. 2013 ;8(5).

38. Lehmann J. The Treatment of Tuberculosis in Sweden with Para-Aminosalicylic Acid (PAS): A Review. Dis Chest. 1949; 16(6):684–703.

39. Fox W, Sutherland I. A five-year assessment of patients in a controlled trial of streptomycin, para-aminosalicylic acid, and streptomycin plus para-aminosalicylic acid, in pulmonary tuberculosis1. Qjm. 1956; 25(2):221–244.

40. Peloquin CA. Therapeutic drug monitoring in the treatment of tuberculosis. Drugs. 2002; 62(15):2169–2183.

41. Donald PR, Diacon AH. Para-aminosalicylic acid: The return of an old friend. Lancet Infect Dis. 2015;15(9):1091–1099.

42. Rengarajan J, Sassetti CM, Naroditskaya V, Sloutsky A, Bloom BR, Rubin EJ. The folate pathway is a target for resistance to the drug para-aminosalicylic acid (PAS) in mycobacteria. Mol Microbiol. 2004; 53(1):275–282.

43. Liwa AC, Schaaf HS, Rosenkranz B, Seifart HI, Diacon AH, Donald PR. Para-aminosalicylic acid plasma concentrations in children in comparison with adults after receiving a granular slow-release preparation. J Trop Pediatr. 2013; 59(2):90–94.

44. PASER® GRANULES Aminosalicylic Acid Delayed-Release Granules. Princeton, NJ. Jacobus Pharmaceutical Company, Inc. 2018; 1–5. Available from: https://www.rxlist.com/paser-drug.htm.

45. Kibleur Y, Brochart H, Schaaf HS, Diacon AH, Donald PR. Dose regimen of para-aminosalicylic acid gastro-resistant formulation (PAS-GR) in multidrug-resistant tuberculosis. Clin Drug Investig. 2014; 34(4):269–276.

46. Mathys V, Wintjens R, Lefevre P, Bertout J, Singhal A, Kiass M, et al. Molecular genetics of para-aminosalicylic acid resistance in clinical isolates and spontaneous mutants of Mycobacterium tuberculosis. Antimicrob Agents Chemother. 2009; 53(5):2100–2109.

47. Nopponpunth V, Sirawaraporn W, Greene PJ, Santi D V. Cloning and expression of Mycobacterium tuberculosis and Mycobacterium leprae dihydropteroate synthase in Escherichia coli. J Bacteriol. 1999; 181(21):6814–6821.

48. Arbex MA, Varella MDCL, Siqueira HR De, Mello FAF De. Antituberculosis drugs: Drug interactions, adverse effects, and use in special situations. Part 2: Second-line drugs. J Bras Pneumol. 2010; 36:626–640.

49. Ratledge C. Iron, mycobacteria and tuberculosis. Tuberculosis. 2004; 84(1–2):110–130.

50. Ratledge C, Brown KA. Inhibition of mycobactin formation in Mycobacterium smegmatis by p-aminosalicylate: a new proposal for the mode of action of p-aminosalicylate. Am Rev Respir Dis. 1972; 106(5):774–776.

51. Wan SH, Pentikainen PJ, Azarnoff DL. Bioavailability of aminosalicylic acid and its various salts in humans III: Absorption from tablets. J Pharm Sci. 1974; 63(5):708–711.

52. Berning SE, Peloquin CA. Antimycobacterial agents: para-aminosalicylic acid. Antimicrob Chemother Balt MD, Williams Wilkins. 1998; 663–668.

53. McEvoy GK. AHFS drug information, 2000. American Society of Health-System Pharmacists; 2000.

54. Iwainsky H. Mode of action, biotransformation and pharmacokinetics of antituberculosis drugs in animals and man. In: Antituberculosis drugs. Springer; 1988, 399–553.

55. Badenhorst CPS, van der Sluis R, Erasmus E, van Dijk AA. Glycine conjugation: importance in metabolism, the role of glycine *N* -acyltransferase, and factors that influence interindividual variation. Expert Opin Drug Metab Toxicol. 2013; 1–15.

56. Lehmann J. The role of the metabolism of p-aminosalicylic acid (PAS) in the treatment of tuberculosis. Interaction with the metabolism of isonicotinic acid hydrazide (INH)

and the synthesis of cholesterol. Scand J Respir Dis. 1969; 50(3):169-185.

57. Bang HO, Jacobsen LK, Strandgaard E, Yde H, others. Metabolism of isoniazid and para-amino salicylic acid (PAS) in the organism and its therapeutic significance. Acta Tuberc Pneumol Scand. 1962; 41(4):237–251.

58. Peloquin CA, Henshaw TL, Huitt GA, Berning SE, Nitta AT, James GT. Pharmacokinetic evaluation of para-aminosalicylic acid granules. Pharmacother J Hum Pharmacol Drug Ther. 1994; 14(1):40–46.

59. Peloquin CA, Berning SE, Huitt GA, Childs JM, Singleton MD, James GT. Once-daily and twice-daily dosing of p-aminosalicylic acid granules. Am J Respir Crit Care Med. 1999; 159(3):932–934.

60. Peloquin CA, Zhu M, Adam RD, Singleton MD, Nix DE. Pharmacokinetics of para-aminosalicylic acid granules under four dosing conditions. Ann Pharmacother. 2001; 35(11):1332–1338.

61. De Kock L, Sy SKB, Rosenkranz B, Diacon AH, Prescott K, Hernandez KR, et al. Pharmacokinetics of para-aminosalicylic acid in HIV-uninfected and HIV-coinfected tuberculosis patients receiving antiretroviral therapy, managed for multidrug-resistant and extensively drugresistant tuberculosis. Antimicrob Agents Chemother. 2014; 58(10):6242–6250.

62. Way EL, Smith PK, Howie DL, Weiss R, Swanson R. The absorption, distribution, excretion and fate of para-aminosalicylic acid. J Pharmacol Exp Ther. 1948; 93(3):368–382.

63. Peloquin CA. Therapeutic drug monitoring: principles and applications in mycobacterial infections. Drug Ther New York. 1992; 22:31.

64. Dheda K, Shean K, Zumla A, Badri M, Streicher EM, Page-Shipp L, et al. Early treatment outcomes and HIV status of patients with extensively drug-resistant tuberculosis in South Africa: a retrospective cohort study. Lancet. 2010; 375(9728):1798–1807.

65. Pietersen E, Ignatius E, Streicher EM, Mastrapa B, Padanilam X, Pooran A, et al. Long-term outcomes of patients with extensively drug-resistant tuberculosis in South Africa: a cohort study. Lancet. 2014; 383(9924):1230–1239.

66. Gandhi NR, Andrews JR, Brust JCM, Montreuil R, Weissman D, Heo M, et al. Risk factors for mortality among MDR-and XDR-TB patients in a high HIV prevalence setting. Int J Tuberc Lung Dis. 2012; 16(1):90–97.

67. Chakraborty S, Gruber T, Barry CE, Boshoff HI, Rhee KY. Para-aminosalicylic acid acts as an alternative substrate of folate metabolism in Mycobacterium tuberculosis. Science. 2013; 339(6115):88–91.

68. Bridge E V, Carr DT, Karlson AG. The effect of a single daily dose of NaPAS on the emergence of streptomycin-resistant strains of tubercle bacilli. In: 12th Conference on the Chemotherapy of tuberculosis. 1953; 9–12.

69. Marsden JA, others. The Single Daily Dose of Sodium Para-Aminosalicylic Acrd usei in conjunction wish Streptomycin in Tuberculosis. Med J Aust. 1954; 2(24):934–935.

70. Yeu WY, Cohen SS. Toleration and Absorption of Sodium Para-Aminosalicylate and Para-Aminosalicylic Acid (Neopasalate). Dis Chest. 1966; 49(2):165–174.

71. Bang HO, Strandgaard E, others. Continued Studieson the Problems of PAS Dosage. Acta Tuberc Scand. 1960; 39(2):8–96.

72. Sy SKB, De Kock L, Diacon AH, Werely CJ, Xia H, Rosenkranz B, et al. N-acetyltransferase genotypes and the pharmacokinetics and tolerability of para-aminosalicylic acid in patients with drug-resistant pulmonary tuberculosis. Antimicrob Agents Chemother. 2015; 59(7):4129–4138.

73. Vasbinder E, van der Weken G, Vander Heyden Y, Baeyens WRG, Debunne A, Remon JP, et al. Quantitative determination of p-aminosalicylic acid and its degradation product m-aminophenol in pellets by ion-pair high-performance liquid chromatography applying the monolithic Chromolith Speedrod RP-18e column. Biomed Chromatogr. 2004; 18(1):55–63.

74. Blumberg HM, Burman WJ, Chaisson RE, Daley CL, Etkind SC, Friedman LN, et al. American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America: treatment of tuberculosis. Am J Respir Crit Care Med. 2003; 167(4):603–662.

75. Campbell Jr EW, Smith MR. Hematology for primary care physicians. Disease-a-Month. 1996; 42(3):129–194.

76. McIlleron H. Tuberculosis drug information. In: Tuberculosis. Elsevier; 2009; 958–971.

77. Berté SJ, DiMase JD, Christianson CS. Isoniazid, Para-aminosalicylic Acid, and Streptomycin Intolerance in 1,744 Patients: An Analysis of Reactions to Single Drugs and Drug Groups plus Data on Multiple Reactions, Type and Time of Reactions, and Desensitization. Am Rev Respir Dis. 1964; 90(4):598–606.

78. Lauener H, Favez G, others. The inhibition of isoniazid inactivation by means of PAS and benzoyl-PAS in man. Am Rev Respir Dis. 1959; 80(1):26–37.

79. Vatsis KP, Weber WW, Bell DA, Dupret J-M, Evans DA, Grant DM, et al. Nomenclature for N-acetyltransferases. Pharmacogenetics. 1995; 5(1):1–17.

80. Zhangwei X, Jianming X, Qiao M, Xinhua X. N-Acetyltransferase-1 gene polymorphisms and correlation between genotype and its activity in a central Chinese Han population. Clin Chim acta. 2006; 371(1–2):85–91.

81. Sim E, Lack N, Wang C-J, Long H, Westwood I, Fullam E, et al. Arylamine N-acetyltransferases: structural and functional implications of polymorphisms. Toxicology. 2008; 254(3):170–183.

82. Kawamura A, Graham J, Mushtaq A, Tsiftsoglou SA, Vath GM, Hanna PE, et al. Eukaryotic arylamine N-acetyltransferase: investigation of substrate specificity by high-throughput screening. Biochem Pharmacol. 2005; 69(2):347–359.

83. Hughes NC, Janezic SA, McQueen KL, Jewett MAS, Castranio T, Bell DA, et al. Identification and characterization of variant alleles of human acetyltransferase NAT1 with defective function using p-aminosalicylate as an in-vivo and in-vitro probe. Pharmacogenetics. 1998; 8(1):55–66.

84. Badawi AF, Hirvonen A, Bell DA, Lang NP, Kadlubar FF. Role of aromatic amine acetyltransferases, NAT1 and NAT2, in carcinogen-DNA adduct formation in the human urinary bladder. Cancer Res. 1995; 55(22):5230–5237.

85. Minchin RF, Hanna PE, Dupret J-M, Wagner CR, Rodrigues-Lima F, Butcher NJ. Arylamine N-acetyltransferase I. Int J Biochem Cell Biol. 2007; 39(11):1999–2005.

86. Hein DW. Molecular genetics and function of NAT1 and NAT2: Role in aromatic amine metabolism and carcinogenesis. Mutat Res - Fundam Mol Mech Mutagen.

2002; 506–507:65–77.

Chapter Two

Literature Review -Analytical Techniques

2.1. Introduction

Separation of analytes from potential interferences is fundamentally important in bio analytical investigations. Additionally, there is a need to determine trace quantities, where the analyte concentration relative to the sample matrix components may be exceptionally small. At current, ultra-high performance liquid chromatography (UHPLC) used as separation method coupled to a mass spectrometer (MS) as the quantification method is widely used in biological and clinical research settings. Together these two systems form a UHPLC-MS/MS technique, which enhances the properties of selectivity, sensitivity and rapid analysis when monitoring therapeutic agents.

This chapter outlines the analytical techniques related to the study presented. Firstly, an overview of basic chromatography is discussed, followed by an elaborate focus on liquid chromatography separation methods, as well as appropriate mass spectrometry detectors. Thirdly, we cover the fundamentals of method development and validation as stipulated by the Food and Drug Administrations (FDA). Fourthly, we discuss the importance of sample preparation and use of internal standards. Lastly, we give a literature overview of LC-MS methodology applied to anti-TB chemotherapeutic PAS and its metabolites APAS and GPAS.

2.2. Description of chromatography

Chromatography is defined as a separation method, which involves two phases – one stationary and one mobile. Separation occurs by transporting the sample or analyte, in the mobile phase through or over a non-miscible stationary phase, which is fixed on a solid

surface or in a column. The sample is dissolved in the mobile phase, which may be a gas (gas chromatography), a liquid (liquid chromatography) or a supercritical fluid (supercritical fluid chromatography).

Components of the sample separate between the two phases, resulting in different migration rates through the system. With the flow of the mobile phase, those components that are retained weakly by the stationary phase elute from the system before strongly retained components. Due to the differences in mobility, components may be divided into discrete bands and be distinctly quantified.[1–3]

Separation modes are distinguished by the physical means in which the analyte and the stationary and mobile phase interacts with each other. More importantly, the classification is based on the types of mobile and stationary phases and the state of equilibrium in the transfer of components between the two phases. The most frequently applied column chromatography has a stationary phase, which is held in a tube, commonly known as a column, through which the mobile phase is applied, by pressure or gravity. The separation method employed relies on the molecular characteristics related to partition (liquid-liquid), adsorption (liquid-solid), affinity (shape) or differences among the molecular weight, as described in **Table 2.1**.[3]

Table 2.1: Summary of common column chromatographic separation methods.

Molecular characteristic	Physical property	Separation method
Polarity	Volatility	Gas-liquid chromatography
	Solubility	Liquid-liquid chromatography
	Adsorptivity	Liquid-solid chromatography
Ionic	Charge	Ion-exchange chromatography
		Electrophoresis
Size (mass)	Diffusion	Gel permeation chromatography
		Dialysis
Shape	Sedimentation	Ultracentrifugation
	Liquid binding	Affinity chromatography

Adapted from Skoog *et al*.[4]

2.3. Chromatographic separation factors

The essential objectives of a chromatographic separation are to obtain optimal resolution within a minimum expenditure of time. Resolution (R_s), of a column provides a quantitative measure of the columns ability to resolve two solutes (analytes), in a mixture. Insufficient R_s, could cause co-elution of analyte, which ultimately decreases the reproducibility of the separation method. Below we discuss the factors related to R_s, namely retention factor (k), selectivity (α), and column efficiency, as displayed in **Figure 2.1**.[4–7]

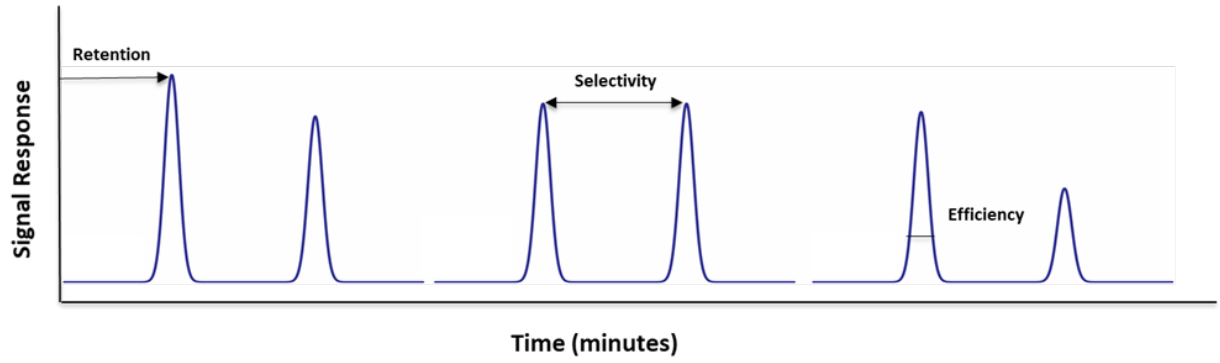

Figure 2.1: Schematic representation of the contributing parameters in chromatographic separations. Adapted from Chromacademy Science.[6]

2.3.1. Retention factor

The retention factor (k), previously known as capacity factor, is the ratio of the time the analyte resides in the column relative to the time it resides in the mobile phase, while the retention time refers to the total time the solute spends in the column. This factor relies on variables such as the flow rate of the mobile phase, and the dimensions of the column.[5,6] The fast elution of an analyte causes the retention time to vary, creating a greater probability of co-elution of the analyte with other components of the sample mixture. Consequently, isobaric interferences occur which are either completely or partially masking the peak of interest, ultimately reducing the analyte response. Alternatively, peak broadening and lengthy run times occurs as a result of too much retention. Under these circumstances, the precision and accuracy in integrating peaks is compromised.[5,8] Optimization of the retention factor can be achieved by altering the mobile phase, stationary phase or phase ratio.

2.3.2. Selectivity

The selectivity factor, also known as separation factor (α) is the ability of the chromatographic system to chemically differentiate the components of the sample mixture. This factor is measured as a ratio of the retention (capacity) factors (k) of the two peaks in question, and is visualized as the distance between the apices of the two peaks.

The selectivity factor should always be larger than 1, as α being 1 is indicative of co-eluting peaks. Therefore, large selectivity values represent good separation between the apex of each peak, and therefore good separation power of the method.[6] Due to dependency on the chemistry of the analyte, the optimization of the selectivity of a separation can also be achieved by modifying the stationary and mobile phase constituents.

2.3.3. Column efficiency

The efficiency of a chromatographic peak is defined by the measure of the peak width and sharpness, as the analyte band travels through the system and column. Ideally, chromatographic peaks would be as sharp as a pencil thin line, but due to dispersion effects, the narrow peaks broaden during their movement through the column. A quantitative measure of chromatographic efficiency is described by, plate number (N).[6]

2.4. Liquid chromatography

This study focused on the use of liquid chromatography (LC), a separation technique ideally suited for molecules of biomedical research, which are usually limited by sample volatility and thermal stability.[7] LC refers to any chromatographic technique in which the mobile phase is liquid, as opposed to gas chromatography (GC), where the mobile phase is a gas.[7] In classical LC, the liquid mobile phase containing the analyte (protein, metabolite, drug) moves through a column, by applying a gravitational force, which causes fractions to be collected over a several hours. The column is discarded after a single separation, and thus needs to be repacked for each separation.[7,9]

Modern LC, also called high performance liquid chromatography (HPLC) has proven to be the predominant technology used in laboratories globally, for decades.(9) HPLC provides the advantage of rapid and superior separations, using high pressure pumps as well as special columns and column packing.[7] The high pressured pumps allowed for a desired flow rate of the solvent, through the column. The selectivity was enhanced by the interaction of both mobile and stationary phase (unique column packing) with the sample.[9]

2.4.1. HPLC instrumentation

A typical UHPLC system performs separation by pumping the mobile phase from a reservoir, by high pressure tubing and fittings, through the column, and out to the detector (**Figure 2.2**). The injector is able to introduce the dissolved sample into the flowing mobile phase stream, which carries the sample to the column. Here, the separation of the molecules occurs

according to the column packing material of the stationary phase and its interaction with the mobile phase. A signal is detected for the separated molecule bands as they elute from the column. A suitable data acquisition system is then used to illustrate a chromatogram, which is generated by plotting the signal intensity as a function of time. High-pressure tubing and fittings are used throughout the system, to interconnect the pump, injector, column and detector components to form the channel for the mobile phase, sample, and separated compound bands.[10] The most critical instrumentation consists of the column and detector.

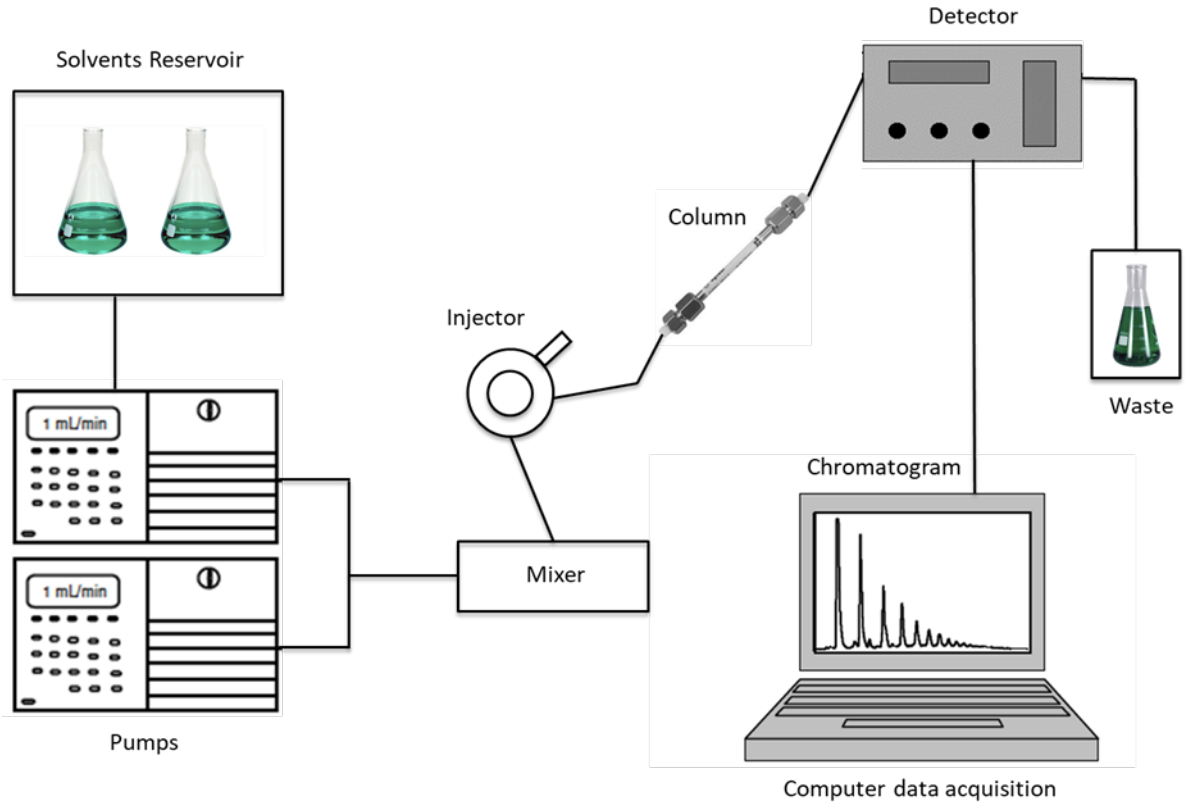

Figure 2.2: A schematic of a typical HPLC system. Adapted from Waters Corporation.[10]

2.4.2. Modes of column separation in LC

The separation principles applied to LC, is dependent of the physico-chemical properties of the analytes of interest. Three primary properties determined prior to separations, include the polarity, electrical charge and molecular size of the analytes. A number of separation mechanisms used, include adsorption chromatography, partition chromatography, and ion exchange. Only the two primary separation mechanisms used, when considering the polarity of the compounds of interest, is discussed below.[10]

2.4.2.1. Normal phase chromatography

Normal phase LC, is a close parallel to adsorption and partition chromatography, which uses a polar stationary phase with a relatively non-polar mobile phase.[1] This technique is commonly referred to as 'liquid-solid' chromatography. In NP-LC, the polar stationary phases such as bare silica or support bonded phases that have a diol, cyano, diethylamino, amino or diamino functional groups are used. Together with these stationary phases, non-polar or weakly polar organic solvents such as hexane, isopropanol, dichloromethane or ethyl acetate are utilized.[7] NP-LC performs separation based on the polar absorption of the molecules in question, or the solvent molecules onto the column surface. The stationary phase is thus very selective for polar (hydrophillic) molecules, which are retained longer while the least polar (hydrophobic) molecules are eluted first.[6,11] Consequently, the highly polar nature of the stationary phase used in NP-LC, may lead to strong binding of polar components present in the mobile phase, ultimately resulting in long column equilibration times and poor reproducibility.[12]

2.4.2.2. Reversed phase chromatography

Reversed phase LC, is a form of partition chromatography, carried out on columns with a non-polar or weakly polar stationary phase. Frequently used stationary phases possess modified silica bearing an *n*-alkyl hydrophobic ligand such as octadecyl (C_{18}), octyl (C_8), *n*-butyl (C_4), phenyl, or cyano-propyl functional groups chemically bonded to microporous silica particles on the column. The type of n-alkyl ligand significantly influences the retention of analyte molecules and thus manipulates the selectivity of the separation.[13] Along with these stationary phases, is the use of a relatively polar mobile phase, usually composed of methanol, acetonitrile, and tetrahydrofuran organic solvents used in different combinations with water.[7] The separation mechanism of RP-LC occurs by partitioning of the solute molecule between the mobile phase and the immobilised non-polar (hydrophobic) stationary phase, in turn causing non-polar molecules to be retained longer than polar molecules. The solutes are therefore eluted in order of increasing molecular hydrophobicity.[13]

However, the elution order on RP-LC varies in respect to factors such as polarity, organic solvent composition, operating temperature, the ionic modifier and gradient elution.[14] Elution can proceed either by isocratic conditions, where the concentration of organic solvent is constant, or by gradient elution whereby the amount of organic solvent is increased over a period of time. For these reasons, RP-LC consists of many factors that can be adjusted in order to affect how an analyte molecule will interact with both the stationary and mobile phase. The hydrocarbon-like makeup of the stationary phase has shown to equilibrate rapidly with these modifications.[13] Other benefits of RP-LC includes a wide range of chromatographic conditions for closely related molecules while also structurally distinct, generally high recoveries, and superior reproducibility of repeated separations carried out

over a long period of time as a result of the stability of the column packing material under a wide range of mobile phase conditions.[15,16]

Both normal and reversed phase chromatography are used for the separation, identification and quantification of anti-TB agents, and their metabolites. Due to its versatility, robustness and high resolving power, RP-LC is mostly utilized in assays developed for anti-retroviral and anti-TB studies. The standard care in TB treatment is combination therapy, and therefore a single separation method is needed to accurately determine a number of compounds of interest. Silica based RP columns which contain di-functionally bonded C_8 and C_{18} ligands has shown favourable results in peak shape. The first choice for method development and analysis of small molecules (molecular weight < 2000 Da) are C_{18} RP columns (**Figure 2.3**).

Figure 2.3: Reversed phase HPLC columns. Adapted from Agilent Technologies.[17]

2.4.3. Optimization of chromatographic separation

Varying experimental conditions until the components of a mixture are separated efficiently with a minimum expenditure of time optimizes a chromatographic separation. In seeking optimum conditions for achieving a desired separation, the critical parameters pertaining to **retention (k'), selectivity (α), and efficiency (N)**, may be adjusted. Optimization is thus aimed at altering the relative migration rates of solutes and at reducing peak broadening[18]. Parameters that are optimized include the column temperature, the column chemistry and dimensions, flow rate, pH of the solvent, organic modifier (i.e. methanol or acetonitrile), buffer strength, the mobile phase gradient, wash solutions and injection solvent.[19,20]

2.5. Ultra-high performance liquid chromatography

Ultra-high performance liquid chromatography (UHPLC) is a modified method of HPLC, as it follows the same principles but brings enhanced performance of separation. The UHPLC method comprises high pressure pumps and small porous particle sizes, which improve run times and separation power.[21]

Researchers have found that a decrease in particle size (sub 2 micron) of the analytical column shows significant increase in the separation efficiency in packed columns. This is a result of small particles providing a more uniform solvent flow rate through the column. In turn, this method allows the mobile phase consumption to be reduced by at least 80% compared to the HPLC, thus shortening the run times up to 7 fold.[22,23]

However, the reduction in particle size leads to a rapid increase in back pressure, due to small particles having a high resistance to solvent flow. Therefore, UHPLC sized particles require a high pressure of up to 6000 psi, to maintain retention and capacity similar to HPLC.[24,25]

2.6. Detection

Several detection systems have been developed for the screening, identification and quantification of drug residues in biological matrices. Most common detectors in LC are concentration-sensitive, in that the output signal is a function of the concentrations of eluate (sample) passing from the column to the detector cell. Largely, a detector should be non-destructive, insensitive to mobile phase composition and fluctuating temperatures while also providing a linear response and sensitivity to low concentrations.[1] Most notably are UV, fluorescence, electrochemical and refractive index detectors. In the current study we have used mass spectrometry as detector, and briefly describe the system.

2.6.1. Mass spectrometric detector

Mass spectrometry (MS) detection is currently the most powerful mode in today's market for quantification and identification of complex sample mixtures. MS essentially involves the production, separation and detection of charged species. Due to the fact that only charged species are detectable, the analyte molecules firstly need to be ionized. Thereafter, ions are filtered based on their mass-to-charge (*m/z*) ratios in the mass analyser.[1,26,27] The relative abundance of the molecule can then be extrapolated from a mass spectrum, which is a 2D plot of *m/z* ratio of the molecules respective ions versus the ion intensity or ion abundance.

When comparing MS to other analytical techniques, the advantages in MS are increased sensitivity and specificity. These characteristics result from a combination of the analyser as a (*m/z*) ratio filter and the ability to determine fragmentation patterns of analyte molecules.[28] For these reasons, MS has become the most ideal detector for interfacing with liquid chromatography systems.

2.7. Hyphenated LC-MS technique

The combination of liquid chromatography (LC) and mass spectrometry (MS), hyphenated LC-MS, combines the separating power of LC, with the strong detection power of MS. This superior technique is particularly valuable in trace level analysis. Depicted in **Figure 2.4** are the basic components of an LC-MS system.

Historically MS had been of limited use in clinical disciplines, due to the need for separation of the analyte molecule from the sample matrix (blood, urine, plasma). Additionally, was the need to produce stable volatile derivatives of the analyte molecule while also subjecting it to ionization. Another limitation of the coupling technique, is the enormous mismatch between the relatively large mass flows involved in LC and the vacuum requirements of the MS. To overcome this problem, several ionization interfaces have been developed for efficient sample introduction into the MS.

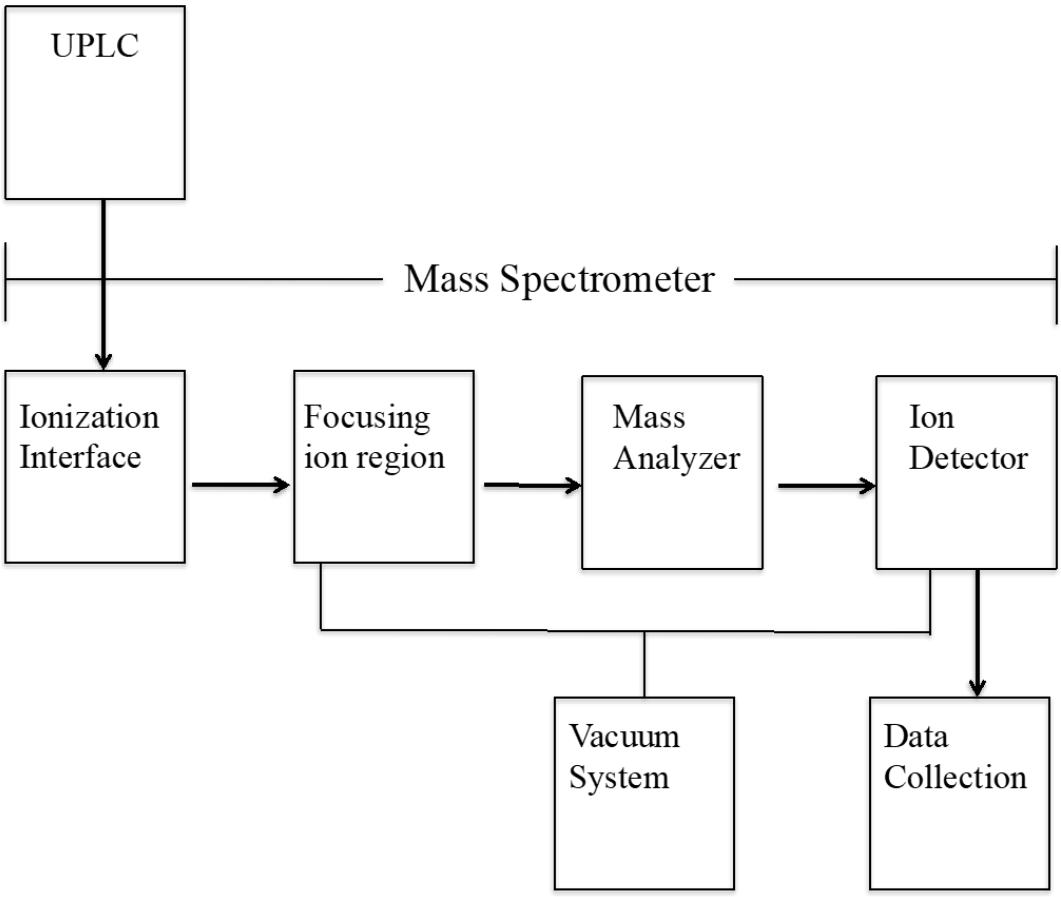

Figure 2.4: Basic components of an UHPLC-MS system. Adapted from Alberts.[18]

2.7.1. Ionization source

MS techniques are distinguished based on the ionization source selected. The choice of source is dependent on its compatibility with the introduction separation method (LC, GC), as well as the resolution of the instrument with the targeted mass range of the analytes.[9] Within the context of this thesis, aspects of the source electrospray ionisation (ESI) are briefly described.

ESI is a form of atmospheric pressure ionization (API) interface, primarily used for the analysis of charged species. API allows ion formation at atmospheric pressure instead of in the vacuum chamber of the mass spectrometer. This enables the direct introduction of non-volatile, charged and unstable analytes into the mass analyzer. It is required that analytes be in the ionized form prior to transfer into the ESI interface, by manipulation of LC eluent pH before or after separation in the column.[26,27] Essentially, three processes are involved in transferring sample ions from the eluent into the gas phase within the instrument. ESI does this as follows. Firstly, the LC eluate is transferred from the column into a metal capillary inlet, which is charged with a high voltage. The action of this high energy at the capillary, together with surrounding high speed gases in the API region, results in production of charged droplets. The droplets are then evaporated using a heated drying gas within the interface, which allows desolvation and increased charge density of the droplets. The repeated action of evaporation produces smaller highly charged droplets, which are capable of forming gas-phase ions that are then analysed in the mass spectrometer by their m/*z* ratios.[27] Therefore, ESI is considered a very low energy ionization process, which usually yields an intact parent ion with single or multiple charges. The charge on the droplet surface depends on the capillary voltage, where a positive capillary forms positive ions (protonated species), and a negative capillary forms negative ions (deprotonated species). Ions that are oppositely charged to the analyser will freely move through a narrow stream that is focused on the mass analyser.[18,26]

2.7.2. Mass analyser

Many types of mass analysers are associated with routine LC-MS analysis, which differ in the fundamental way they separate species. The most commonly used designs include quadrupole, ion trap, orbitrap and time-of-flight analysers.

The quadrupole mass analyser contains four parallel rods arranged in a symmetrical manner. The opposing rods are connected together by applying controlled voltages to the rods, which impart an electrostatic/magnetic field inside the analyser. The ions produced in the source, are focused into the analyser, and travel along the axis of the rods. For a specific range of voltages applied, only ions with a certain m/*z* ratio will pass through the quadrupole without touching the rods, therefore these ions are known to have stable trajectories. Ions with unstable trajectories will collide with a rod and become neutralized. Subsequently, these ions will not enter the detector. Hereby, separation is achieved by controlling the voltages applied to the mass analyser rod, so that ions with varying m/*z* ratios can be focused onto the detector for their MS spectrum to be generated.[4,27]

The golden standard of quadrupole mass analysers is known as the triple-quadrupole mass analyser. This system contains three sets of quadrupole rods arranged in series, for the combination of two mass selective experiments. In the first quadruople (Q1), ions of a particle *m/z* ratio are filtered, known as the precursor ion. The second quadrupole (nowadays the quadrupole is replaced by a hexapole or other device) is a collision induced dissociation (CID) source, consisting of an inert gas such as argon or helium, used to fragment the

precursor ions from Q1. The third quadrupole (Q3) filters fragment ions from the CID source, known as the product ions. The result is known as tandem mass spectrometry (MS/MS).[26]

2.8. Storage of biological samples

In order to avoid degradation or other potential chemical changes in the analyte drug, the biological sample is usually frozen immediately upon collection and thawed before analysis. In most cases, pre-treatment of sample is necessary before running the assay. The appropriate method of preparation depends on factors such as texture, chemical composition of the sample and the degree of drug protein binding. Usually, the sample stability is tested prior to collection to determine a protocol for this procedure.

2.9. Sample preparation

The advantage of combining LC with MS has shown to provide an analytical technique with a high capability to distinguish between different molecules. However, despite the advances made in instrumentation for determining analytes in biological samples, there is still a need for pre-treatment of the sample. The purpose of sample preparation includes the following: removal of interfering endogenous biological substances that may lead to ion suppression, which causes these substances to co-elute with the analyte from the LC column. Secondly, is the need to convert the analyte into a more suitable form for injection, separation and detection. Thirdly, is to concentrate the target analyte to improve the sensitivity.[5,29]

Several preparation techniques have been developed, the most common liquid phase methods applied to LC-MS instruments include liquid-liquid extraction (LLE), solid phase extraction (SPE), and protein precipitation (PPT). For some analyte molecules, precipitation may be the first step, followed by purification and concentration by LLE or SPE.[29] An advantage in liquid samples, usually found in biological studies, is their ability to dissolve in any polar or non-polar organic solvent.[5] In this study, we have applied PPT as it is widely used for plasma and blood sample extraction.

2.9.1. Protein precipitation (PPT)

This simple, cost effective and rapid preparation method is used for the clean-up of analytes from a sample with a relatively high abundance of protein. It is therefore, suitable for extraction of biological samples (plasma, serum, urine and blood) in clinical, toxicological and therapeutic drug monitoring (TDM) studies. Its cost effectiveness, simplicity and rapidity has made PPT the most frequently employed technique for analysis of anti-TB agents, as they are hydrophobic in nature.[30]

The technique is based on the interaction between the reagent applied and the moieties of the present proteins. Reagents such as water miscible organic solvents, interfere with the intramolecular hydrophobic interactions of proteins while also reducing the hydration state of the proteins. Consequently, the less hydrated proteins are, the less soluble they become.

A typical PPT protocol is as follows. An organic solvent, commonly acetonitrile, acetone or methanol, is added to the biological sample. Following the precipitation reaction, which may require vortexing (mixing), the sample mixture is centrifuged to draw the protein precipitate to the bottom of the sample tube. The analyte separated to the top liquid layer, is then removed or evaporated to dryness and reconstituted in a more suitable solvent for injection into the LC-MS. The instrumental response of the analyte in the unknown sample is then obtained from a sample in which the concentration is known.[5,30] This technique does not get rid of lipids and small polar molecules like anions, cations, amino acids and sugars and it should be kept in mind when the chromatographic methods are developed to separate the analytes from these molecules that might interfere in the analysis.

2.10. Calibration and Internal standards

In order to maintain reliable quantitative data, the LC-MS system compares the instrumental response obtained for the analyte in the unknown clinical sample with that from a sample with a known concentration, referred to as a calibration standard. In typical study setting, a set of calibration standards (usually at least 6 points) are prepared over the target concentration range of the analyte of interest. The instrumental response of each calibrator is measured and the relationship between response (y-axis) and known concentrations of calibrators (x-axis) is established. Typically, the relationship should be linear, as shown in **Figure 2.5**. This calibration curve is then used to transform measurements made on test samples into estimates of the amount of analyte present in a clinical sample.[5,31] The range of calibrators include the limit of detection (LOD), which is the lowest concentration at which the analyte is detectable but not necessarily quantifiable as an exact value. Also in the range is the lower limit of quantification (LLOQ), presenting the lowest concentration at which the

analyte can be quantified with acceptable precision (repeatability and reproducibility) and accuracy under the specified conditions of analysis.

Routinely, LC-MS methods use calibrators processed in the same manner as the clinical samples. Calibrators are subjected to the same type of matrix, which is known to be free of the analyte, extracted with the same technique and at the same time.

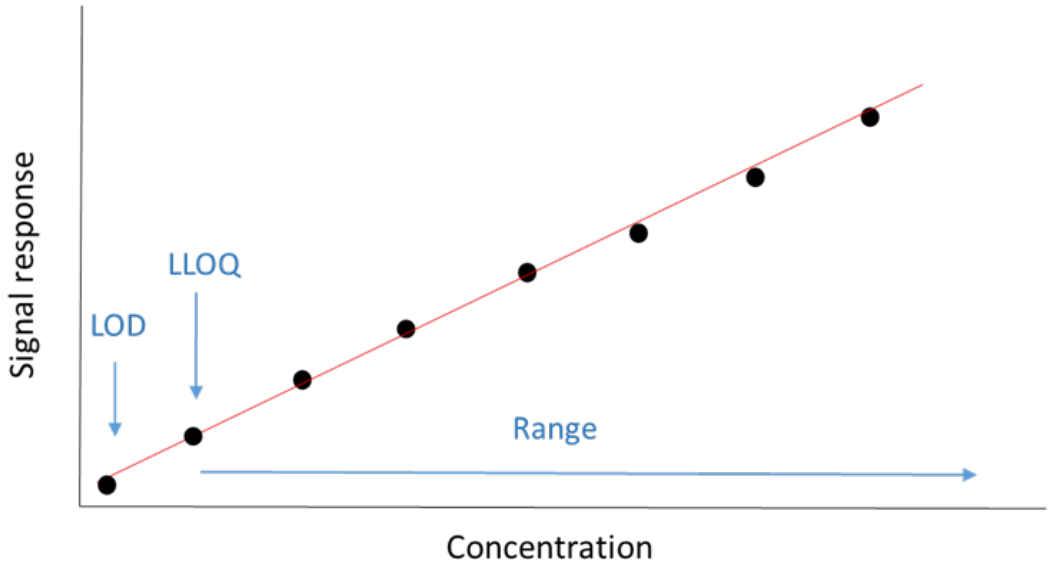

Figure 2.5: Typical calibration curve. Position of limit of detection (LOD) and lower limit of quantification (LLOQ) within the concentration range, are indicated. Adapted from Barwick.[31]

Another way of ensuring reproducibility and reliability is the incorporation of a known amount of a non-analyte compound, named an internal standard (IS), to clinical samples, calibration standards and blank samples prior to extraction and analysis. Hereby, the calibration curve illustrated in **Figure 2.5**, presents the known concentrations of analyte (x-axis) versus the ratio of analyte response to the internal standard response (y-axis). By adding an IS, the analyst is able to identify differences in extraction from sample to sample, and to

correct for effects such as instrument drift or variable sample injection volume. This is possible because the IS is added to each sample and therefore measured in the same solution and at the same time. It is however necessary to use an IS which has similar chromatographic behaviour as the analyte of interest, while still being distinguishable from the analyte in the mass spectrum.[5]

Quality control samples (QC's) are samples that have known concentrations of the analyte that are analysed in between the test samples to ensure that the system stays accurate throughout the run. Typically three QC's are made by fortification of a plasma/matrix blank sample at three different levels, one just above the lower limit of quantification one at an intermediate concentration and one just below the higher limit of quantification, designated LOQ, MQC and HQC respectively. Acceptance of the data relies on QC's being successfully quantified within predefined limits, determined by method validation criteria for bioanalytical research.[5]

2.11. Method validation

In LC, validation is the process of producing documented evidence that components such as the instrument and method perform as expected within specified design parameters and requirements. This is to ensure that the obtained results are reliable with respect to the equipment, including the instrument and computer controlling it, and the analytical method used to run on that equipment.

After development of an analytical method, the process of validation is established by laboratory studies and performance characteristics, as a tool of quality assurance.[32] This is to ensure that the method meets the requirements for the intended analytical application. **Table 2.2** describes the performance parameters, which have been evaluated in this study, according to guidelines stipulated by the Food and Drug Administration.

During method development the stability of the analyte in the given matrix, which includes the effects of sample collection, handling and storage, is a prerequisite for reliable quantification.[33,34] This is to ensure that the concentration of the analyte at the time of sample collection corresponds with the concentration at the time of analysis.[35] Stability should be assessed in the same matrix as intended for the in study samples, and under typically expected sample conditions as described in **Table 2.3**. Comparing the experimental standard solution response relative to a freshly prepared standard tests the stability parameter. Acceptance criteria includes a consistent RSD of 2% and 20% at the standard stock solutions and the LLOQ, respectively.

Table 2.2: Acceptance criteria of various method validation parameters.[33]

Parameter	Experimental procedure	Acceptance criteria
Specificity	3 x injection of 3 blank samples from various matrices.	Chromatograms should present no interference from the blank sample.
	Injection of a sample spiked with the analytes of interest.	Chromatograms should present no co-eluting peaks at the same retention time as the analytes.
Precision	Repeated injections of calibration standards for analysis per batch samples.	20% RSD of peak area at LLOQ should not be exceeded. At higher concentrations, 15% RSD is accepted.
Accuracy	5 x injection of spiked QC samples at 3 concentrations (LLOQ, LQC, MQC, HQC).	Accuracy is reported as the % recovery of the difference between mean measured amount and the true value, which should not exceed 20% from the actual value, i.e. 80 – 120% recovery.
Linearity	5 x injection of 6 calibration standards prepared at 25, 50, 75, 100, 150, and 200%	RSD and accuracy % must be reported for each standard. The peak area of each standard should be plotted on a calibration curve using Excel to calculate regression equation and correlation coefficient > 0.999.
Lower limit of quantification (LLOQ)	5 x injection of lowest standard on calibration curve.	Concentration response of 5 x blank sample, with RSD and accuracy % no less than 20%.
Limit of detection (LOD)	5 x injection of standard.	Concentration response of 3 x SD of blank sample is acceptable.
Robustness	Reported by changes in concentration as a response to changes in key variables.	Discretion should be used to decide.
Intra- and inter-day reproducibility	Repeated injections of calibration standards for analysis, per batch on different days (inter-precision) and on the same day between different batches (intra-precision).	20% RSD of peak area at LLOQ should not be exceeded. At higher concentrations, 15% RSD is accepted.

RSD = relative standard deviation; QC's = quality controls; LLOQ = lower limit of quantification; LQC = lower quality control; MQC = medium quality control; HQC = high quality control; SD = standard deviation

Table 2.3: Typical stability experiments evaluated during method development, for various stages of analysis.[33,34]

Stability determinant	Experimental procedure
Freeze-thaw	3 x QCs prepared in triplicate and subjected to 3 x freeze/thaw cycles, to account for stability during reanalysis of samples.
Long term	QCs prepared in intended matrix at the intended storage temperature (i.e. - 80º C) and over the same period.
Auto-sampler	QCs prepared appropriately and subjected to auto-sampler conditions i.e. temperature and expected maximum time of analytical run.
Temperate	QCs prepared at conditions of analyte during sample extraction process (i.e. temperate and time needed for preparation) should be determined to prevent degradation of analyte during sample preparation
Stock solution	QCs prepared under intended storage conditions of sample prior to analysis, i.e. refrigerator (~ 4º C).

2.12. Para-aminosalicylic acid

Para-aminosalicylic acid (PAS, 4-amino-2-hydroxybenzoic acid) is an acid of about the same strength as benzoic acid.[36,37] PAS is approximately 0.2% soluble in water, but its salt formulations are soluble up to 96%.[38] It has a chemical structure comprised of carboxyl, hydroxyl and amine groups. The acetylation of the free amino group and conjugation at the carboxyl group are most prominent steps in the biotransformation process. In man, glycine-PAS (GPAS) presents the main conjugation product, while acetyl-PAS (APAS) is the main acetylated product. The decarboxylation product excreted with the urine under normal conditions, known as m-aminophenol, is another by-product formed non-enzymatically.[39,40]

2.13. Analytical analysis of PAS and major metabolites

Several methods have been developed for the determination of PAS, fewer methods for APAS and only two reported for GPAS in human biological fluids. Among the earlier methods is spectrophotometry and paper chromatography applied by Way *et al.*[41] and Unverricht *et al.*[42] for the analysis of PAS in blood and urine, respectively. For the quantification of free PAS GPAS and APAS in plasma, a simple method was established using a colourimetric route by Lauener *et al.*[43] A similar method (unpublished) was used by Lehmann[39] in whole blood and urine. These methods lacked specificity, as they were severely affected by various medications including antibiotics, sulphonamides and some food ingredients.[44]

The earliest mention of the determination of PAS in plasma using HPLC was reported by Honigberg *et al.*[45] Therapeutic levels were quantified with a non-extractive sample preparation method and an ion-pairing reversed-phase (RP) HPLC technique coupled to a fluorescence detector. The reversed-phase system allowed the mobile phase to be miscibile with the sample matrix, and for the rapid elution of polar components in the matrix. Due to the acidic nature of PAS (pKa = 3.25), the ion-pairing mobile phase was used to increase the factor k, to additionally resolve the drug from components in the matrix. The use of HPLC proved to eliminate the interference from other components, which was a major limitation in the colorimetric assay.

Although analysis by HPLC showed to be more specific, researchers evaluated alternative methods to overcome the complicated equipment and running costs. To directly determine

PAS and p-aminobenzoic acid (PABA) in various biological fluids, Pemberton *et al*.[46] and Liandiou and Ioannou[44] used a spectrofluorometric method. These fluorescence methods provided a cost effective alternative as well as less susceptible to exogenous interferences than the colorimetric method. However, the problem was not entirely solved. The lack of complete specificity still remained, due to several overlapping spectra from endogenous and extraneous compounds.

Cummins *et al*.[47] quantified PAS and acetylated metabolite APAS, in human urine using capillary zone electrophoresis method. Direct injection of urine onto the capillary showed no significant inferences with the exception of aspirin (acetylsalicylic acid, ASA), which eluted very close to the APAS peak.

For the purpose of pharmacokinetic studies, the literature reports many assays established to simultaneously assay second-line anti-tuberculosis (anti-TB) agents, which includes PAS and often its metabolite APAS. These methods include HPLC systems coupled to fluorescence detection[48] and ultraviolet (UV) detection.[49,50] The UV detection methods included an ion interaction reagent and use of different mobile phases, making this technique suitable for simultaneous separation of xenobiotics with a broad range of polarities.

Apart from its use as a second-line anti-TB drug, PAS is considered a promising therapeutic agent for treatment of manganese-induced neurodegenerative disorders. To determine how PAS exerts its therapeutic effectiveness and whether it or its metabolites can cross the blood-brain barrier, Hong *et al*.[51] developed and validated an HPLC method coupled to fluorescence detector to assay PAS and APAS in plasma, cerebrospinal fluid (CSF) and tissues of rat models.

An HPLC method combined with MS detection using electrospray interface was developed by Pastorini *et al*.[52] for the determination of 5-aminosalicylic acid (5-ASA) and its metabolite N-acetyl-5-aminosalicylic acid (N-Ac-5-ASA) in human plasma. This study used PAS and APAS as internal standards, which is the first known mention of analysing PAS and its derivatives with a mass detector. Hereafter, PAS was assayed to determine its pharmacokinetics in plasma samples using a HPLC coupled to a MS/MS detector with an atmospheric turbulon ionization interface. The validated method used another anti-TB agent, Thiacetazone as internal standard.[53] Kim *et al*.[54] reported a similar LC-MS/MS method for the quantification of 20 anti-TB drugs including PAS, in human plasma.

In more recent years, UHPLC-MS/MS has also been utilized in the quantification of first-line and second-line anti-TB agents, in human plasma.[55–57] These methods were uniquely designed for simultaneous analysis of drugs used in the standard treatment regimen of MDR- and XDR-TB patients therefore, making the technique advantageous for the therapeutic drug monitoring of most prescribed anti-TB medications. However, to the best of our knowledge, no method that is applicable simultaneously to PAS and both metabolites (APAS, GPAS) has been developed on a UHPLC-MS/MS system.

A summary of the mentioned analytical techniques developed to quantify PAS alone, and/or simultaneously with other anti-TB agents, is presented in **Table 2.4**.

Table 2.4: Summary of internal standards, columns and solvents reported in the literature for analysis of anti-tuberculosis drug PAS in various matrices, using HPLC and UHPLC methods.

Author	Analyte	Sample volume and matrices	Internal standard	Sample preparation	LC columns	Mobile phase	Range (µg/mL)
(45)	PAS	100 µL human plasma	Anthranilic acid	PPT with 100% Methanol	LiChrosorb C18 column (250 x 3.2 mm; <10 µm)	Methanol:water (20:80, v/v) containing 0.005 M tetrabutyl-ammonium hydroxide and 0.01 M disodium acid phosphate (pH 5.5, adjusted with phosphoric acid)	2.0 – 50.0
(46)	PAS, PABA	100 µL human urine	Not available	Alkaline hydrolysis with 0.5 mL of 4 M sodium hydroxide	Polymer Laboratories 100 A RP-S column (150 x 4.6 mm; 8µm)	Acetonitrile (12%) in 100 mM sodium dihydrogen phosphate buffer (pH 4.6)	0.025 – 15.0 mM
(48)	PAS	Human plasma and urine	Not available	Not available	Lichrospher 100 RP18 column (250 x 4.6 mm; 5 µm)	Not available	1.0 – 100.0 µg
(49)	PAS	Rat serum and plasma	Not available	Filtration through 0.20 µm Millipore filter and 1/20 dilution with ultrapure water	Lichrospher 100 RP18 column (250 x 4.6 mm; 5 µm)	Acetonitrile:water (50:50, v/v) in 100% acetonitrile	Not available
(50)	PAS MAP	Pellets	Acetanilide	PPT and reconstitution with mobile phase	RP 18e column and Chromolith SpeedROD RP-18e column	20 mM phosphate buffer, 20 mM TBAS and 16% (v/v) methanol	Not available
(52)	5-ASA N-5-ASA	Human plasma	5-ASA: para-aminosalicylic acid N-5-ASA: N-acetyl-para-aminosalicylic acid	PPT, and reconstituted with 50 mmol/L acetic acid in 500 µL water	Synergi-Hydro-RP (150 x 2.0 mm; 4 µm)	17.5 mmol/L acetic acid in water (A), acetonitrile (B)	0.05 – 4.0
(51)	PAS APAS	200 µL rat plasma 200 µL rat CSF 200 µL rat brain tissue	5-ASA	PPT with methanol and reconstituted with mobile phase LLE with 6.0 M hydrochloric acid, followed by PPT as described in plasma PPT with methanol and 6.0 M hydrochloric acid, followed by reconstitution with mobile phase	RP Econosphere C18 column (250 x 4.6 mm; 5 µm)	17.5 mM potassium phosphate buffer at pH 3.5 (A), methanol (B)	0.005 – 500 0.017 – 166.7 µg/g 0.017 – 166.7

Table 2.3: Summary of internal standards, columns and solvents reported in the literature for analysis of anti-tuberculosis drug PAS in various matrices, using HPLC and UHPLC methods (continued).

Author	Analyte	Sample Volume and Matrices	Internal Standard	Sample Preparation	LC Columns	Mobile Phase	Range (µg/mL)
(53)	PAS	100 µL human plasma	THIA	PPT with methanol	Agilent Zorbax column (150 x 2.1 mm; 3.5 µm)	0.1% formic acid in water (A), 0.1% formic acid in methanol (B)	1 – 100
(56)	STP KMN CLS PAS CTM PTM MXF LEV LNZ	50 µL human serum	STP: Dihydrostreptomycin KMN: Gentamicin CLS: Muscimol PAS: 4-aminobenzoic acid CTM: Roxithromycin PTM: Ethionamide MOX, LEV, LNZ: Lomefloxacin	PPT with 3 M HCl in 50% methanol, and reconstitution with 1M NaOH in 100% methanol	Waters HSS T3 column (50 x 2.1; 1.8 µm)	10 mM ammonium formate in 0.1% formic acid (A), 0.1% formic acid in acetonitrile (B)	STP, KAN, CLS, PAS: 5.0 – 100.0 CLM, PTM: 0.5 – 10.0 MXF, LEV, LNZ: 1.0 – 20.0
(54)	AKN STP KMN PZA INH RFM ETM CLS PAS LNZ MXF LEV CTM PTM AMX CPX CFM RTM	100 µL human plasma 50 µL human plasma	AKN, STP, KMN, PZA: Apramycin RFM: Rifampin-d_3 INH, ETM, CLS, PAS, LNZ, MXF, LEV, CTM, PTM, AMX, CPX, CFM RTM: Moxifloxacin-d_4	PPT with methanol PPT with acetonitrile	Atlantis HILIC column (150 x 2.1 mm; 3 µm)Atlantis dC18 column (150 x 2.0 mm; 3 µm)	0.1% formic acid (A), 0.1% formic acid in acetonitrile (B)	AKN, KAN, STP: 1.0 – 50.0 PZA: 2.0 – 100.0 AMX, LNZ: 0.4 – 20.0 CPX, LEV, MXF, PTM, RFM, RTM: 0.2 – 10.0 CTM, ETM, ETH, INH: 0.1 – 5.0 CFM: 0.04 – 2.0 CLS: 0.8 – 40.0 PAS: 1.0 – 50.0

Table 2.3: Summary of internal standards, columns and solvents reported in the literature for analysis of anti-tuberculosis drug PAS in various matrices, using HPLC and UHPLC methods (continued).

Author	Analyte	Sample Volume and Matrices	Internal Standard	Sample Preparation	LC Columns	Mobile Phase	Range (µg/mL)
(57)	CLS PAS PTM LEV STP MXF KMN	50 µL human serum	CLS: Muscimol PAS: 4-aminobenzoic acid CTM: Roxithromycin PTM: Ethionamide STP: Dihydrostreptomycin MOX, LEV: Lomefloxacin KMN: Gentamicin	PPT with 3 M HCl in 50% methanol, and reconstitution with 1M NaOH in 100% methanol	HSS T3 column (50 x 2.1 mm; 1.8 µm)	10 mM ammonium formate in 0.1% formic acid (A), 0.1% formic acid in acetonitrile (B)	STP, KMN, CLS, PAS: 5.0 – 100 PTM: 0.5 – 10.0 MXF, LEV: 1.0 – 20.0
(58	STP KMN CTM CLS MXF LEV LNZ PAS PTM	100 µL whole blood	STP: Dihydrostreptomycin KMN: Gentamicin CTM: Roxithromycin CLS: Muscimol MOX, LEV, LNZ: Lomefloxacin PAS: 4-aminobenzoic acid PTM: Ethionamide	PPT with 3 M HCl in 50% methanol, and reconstitution with 1M NaOH in 100% methanol	HSS T3 column (50 x 2.1 mm; 1.8 µm)	10 mM ammonium formate in 0.1% formic acid (A), 0.1% formic acid in acetonitrile (B)	STP, KMN, CLS, PAS: 5 – 100 CTM, PTM: 0.5 – 10 MXF, LEV, LNZ: 1 – 20 ,

PAS – Para-aminosalicylic acid; PABA – 4-aminobenzoice acid; INH – Isoniazid; ETH – Ethionamide; MOR – morphazinamdie; MAP – m-aminophenol; 5-ASA – 5-aminosalicylic acid; N-5-ASA – N-acetyl-5-aminosalicylic acid; APAS – N-acetyl-para-aminosalicylic acid; STP – streptomycin; KMN – kanamycin; CLS – Cycloserine; CTM – clarithromycin; PTM – Prothionamide; MXF – Moxifloxacin; LEV – Levofloxacin; LNZ – Linezolid; AKN – Amikacin; PZA – Pyrazinamide; RFM – Rifampicin; ETM – Ethambutol; AMX – Amoxicillin; CPX – Ciprofloxacin; CFM – Clofazimine; RTM – Roxithromycin

References

1. Encyclopedia of Chromatography 1st ed. (2001). Boca Ranton, Florida, USA, Jack Cazes, 1045.

2. Miller JM, Burns DT. Chromatography: concepts and contrasts (1988). Wiley, New York, USA.

3. Coskun O. Separation Tecniques: Chromatography. North Clin Istanbul. 2016; 3(2):156–160.

4. Skoog DA, Holler FJ, Crouch SR.Principles of Instrumental Analysis 6th ed. (1998). Thomson, Belmont, CA, 429-443.

5. Sargent M. Guide to achieving reliable quantitative LC-MS measurements. Analytical Methods Committee. Teddington, Middlesex, UK. 2013. 1-31.

6. The Theory of HPLC: Chromatographic Parameters. Chromagraphy, Crawford Scientific, New Jersey, USA, 2014. Available from: http://www.chromacademy.com/index.html.

7. Ahuja, S. Chromatography and Separation Science 4th ed. (2003). Academic Press, North Carolina, USA, 1–251.

8. The Theory of HPLC normal phase (absorption) chromatography. Chromacademy, Crawford Scientific, New Jersey, USA, 2014. Available from: http//www.chromacademy.com/index.html.

9. Snyder LR, Kirkland JJ.; Glajch JL. Practical HPLC Method Development (1997). John Wiley and Sons, New York, USA, 1-110.

10. Beginners guide to liquid chromatography, Waters Corporation, Massachusets, USA 2018. Available from: http//www.waters.com/waters/en_US/HPLC---High Performance-Liquid-Chromatography/Explained/nav.htm?cid=10048919&locale=en_US.

11. Snyder LR. Kirkland JJ. Introduction to modern Liquid Chromatography 2nd ed. (1979). John Wiley and Sons Inc, New York, USA1-882.

12. Buszewski B, Noga S. Hydrophilic interaction liquid chromatography (HILIC)-a powerful separation technique. Anal Bioanal Chem. 2012; 402(1):231–247.

13. Aguilar MI. Reversed-phase high-performance liquid chromatography. In: HPLC of Peptides and Proteins. Springer. 2004, p. 9–22.

14. Dorsey JG, Cooper WT, Wheeler JF, Barth HG, Foley JP. Liquid chromatography: theory and methodology. Anal Chem. 1994; 66(12):500–546.

15. Aguilar MI, Hearn MTW. High-resolution reversed-phase high-performance liquid chromatography of peptides and proteins. In: Methods in enzymology. Elsevier; 1996 3–26.

16. Mant CT, Hodges RS. Analysis of peptides by high-performance liquid chromatography. In: Methods in enzymology. Elsevier; 1996, 3–50.

17. HPLC: Resolution , efficiency , selectivity and capacity. Agilent Technologies, California, USA, 2018; 1–2.

18. Alberts P. Development of a novel LC-MS/MS method for the detection of adulteration of South African sauvignon blanc wines with 3-alkyl-2-methoxypyrazines. MSc Thesis, Stellenbosch University. 2008.

19. Sandra P. Resolution - Definition and nomenclature. J High Resolut Chromatogr. 1989; 12(2):82–86.

20. McMaster MC. LC/MS: A practical user's guide. John Wiley & Sons; New York, USA, 2005.

21. Chawla G, Ranjan C. Principle, Instrumentation, and Applications of UPLC: A Novel Technique of Liquid Chromatography. Open Chem J. 2016; 3(1):1–16.

22. Chesnut SM, Salisbury JJ. The role of UHPLC in pharmaceutical development. J Sep Sci. 2007; 30(8):1183–1190.

23. MacNair JE, Lewis KC, Jorgenson JW. Ultrahigh-Pressure Reversed-Phase Liquid Chromatography in Packed Capillary Columns. Anal Chem. 1997; 69(6):983–989.

24. Swartz M. HPLC detectors: A brief review. J Liq Chromatogr Relat Technol. 2010; 33(9–12):1130–1150.

25. Taleuzzaman M, Ali S, Gilani SJ, Imam SS, Hafeez A. Ultra performance liquid chromatography: A review. Austin J Int Anal Pharm Chem. 2015; 2(6):39–44.

26. Dooley KC. Tandem mass spectrometry in the clinical chemistry laboratory. Clin

Biochem. 2003; 36(6):471–481.

27. Mass spectrometry fundamental LC-MS introduction. Chromacademy, Crawford Scientific, New Jersey, USA, 2015. Available from: http://www.ecs.umass.edu/eve/background/methods/chemical/Openlit/Chromacademy LCMS Intro.pdf.

28. Willard HH, Merritt Jr LL, Dean JA, Settle Jr FA. Instrumental methods of analysis 7th ed. (1988).

29. Ashri NY, Abdel-Rehim M. Sample treatment based on extraction techniques in biological matrices. Bioanalysis. 2011; 3(17):2003–2018.

30. Raju KSR, Gundeti M, Malik MY, Kadian N, Rashid M, Taneja I, et al. Bioanalysis of antitubercular drugs using liquid chromatography. J Pharm Biomed Anal. 2017; 134:295–309.

31. Barwick VJ. Preparation of calibration curves: a guide to best practice. Teddington, UK. 2003.

32. Rajpoot BS. Shri RNS. Validation and Process Development: a Review. IRJP. 2001; 2(21):32–39.

33. Validation BM. Guidance for Industry Bioanalytical Method Validation Guidance for Industry Bioanalytical Method Validation. FDA. 2018; 1–22.

34. Peters FT, Drummer OH, Musshoff F. Validation of new methods. Forensic Sci Int. 2007; 165(2–3):216–224.

35. MS, Vijay AR, NS. Bioanalytical Method Development and Validation of Esomepraole in Human Plasma by LCMS/Ms. Asian J Res Chem. 2010; 3(2):477–484.

36. Lehmann J. The treatment of tuberculosis in Sweden with para-aminosalicylic acid; a review. Dis Chest. 1949; 16(6):684–703.

37. Zheng W, Jiang YM, Zhang Y, Jiang W, Wang X, Cowan DM. Chelation therapy of manganese intoxication with para-aminosalicylic acid (PAS) in Sprague-Dawley rats. Neurotoxicology. 2009; 30(2):240–248.

38. Rosdahl GK. Some properties and derivatives of para-amino-sahcylic acid (PAS).

Sven kem Tidskr. 1948; 60:12.

39. Lehmann J. The role of the metabolism of p-aminosalicylic acid (PAS) in the treatment of tuberculosis. Interaction with the metabolism of isonicotinic acid hydrazide (INH) and the synthesis of cholesterol. Scand J Respir Dis. 1969; 50(3):169-185.

40. Iwainsky H. Mode of action, biotransformation and pharmacokinetics of antituberculosis drugs in animals and man. In: Antituberculosis drugs. Springer; 1988; 399–553.

41. Way EL, Smith PK, Howie DL, Weiss R, Swanson R. The absorption, distribution, excretion and fate of para-aminosalicylic acid. J Pharmacol Exp Ther. 1948; 93(3):368–382.

42. Unverricht W, Schattmann K, Senft G. Paper chromatography of isoniazid and p-aminosalicylic acid. Arztl Wochensch. 1954; 9(35):838.

43. Lauener H, Favez G, others. The inhibition of isoniazid inactivation by means of PAS and benzoyl-PAS in man. Am Rev Respir Dis. 1959; 80(1):26–37.

44. Lianidou ES, Ioannou PC. Simple spectrofluorometric determination of p-aminobenzoic and p-aminosalicylic acids in biological fluids by use of terbium-sensitized luminescence. Clin Chem. 1996; 42(10):1659-1665.

45. Honigberg IL, Stewart JT, Clark TC, Davis DY. Non-extractive fluorometric measurement of p-aminosalicylic acid in plasma by ion-pairing techniques and high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl. 1980; 181(2):266–271.

46. Pemberton PW, Gagjee P, Chaloner C, Braganza JM, Lobley RW. Spectrofluorimetric determination of urinary p-aminobenzoic and p-aminosalicylic acids in the BT-PABA/PAS test of pancreatic function. Clin Chim Acta. 1991; 199(3):253–262.

47. Cummins CL, O'Neil WM, Soo EC, Lloyd DK, Wainer IW. Determination of p-aminosalicylic acid and its N-acetylated metabolite in human urine by capillary zone electrophoresis as a measure of in vivo N-acetyltransferase 1 activity. J Chromatogr B Biomed Appl. 1997; 697(1–2):283–288.

48. Peloquin CA, Zhu M, Adam RD, Singleton MD, Nix DE. Pharmacokinetics of para-aminosalicylic acid granules under four dosing conditions. Ann Pharmacother. 2001;

35(11):1332–1338.

49. Gennaro M., Calvino R, Abrigo C. Ion interaction reagent reversed-phase high-performance liquid chromatography determination of anti-tuberculosis drugs and metabolites in biological fluids. J Chromatogr B Biomed Sci Appl. 2001; 754:477–486.

50. Vasbinder E, van der Weken G, Vander Heyden Y, Baeyens WRG, Debunne A, Remon JP, et al. Quantitative determination of p-aminosalicylic acid and its degradation product m-aminophenol in pellets by ion-pair high-performance liquid chromatography applying the monolithic Chromolith Speedrod RP-18e column. Biomed Chromatogr. 2004; 18(1):55–63.

51. Hong L, Jiang W, Zheng W, Zeng S. HPLC analysis of para-aminosalicylic acid and its metabolite in plasma, cerebrospinal fluid and brain tissues. J Pharm Biomed Anal. 2011; 54(5):1101–1109.

52. Pastorini E, Locatelli M, Simoni P, Roda G, Roda E, Roda A. Development and validation of a HPLC-ESI-MS/MS method for the determination of 5-aminosalicylic acid and its major metabolite N-acetyl-5-aminosalicylic acid in human plasma. J Chromatogr B Anal Technol Biomed Life Sci. 2008; 872(1–2):99–106.

53. Liwa AC, Schaaf HS, Rosenkranz B, Seifart HI, Diacon AH, Donald PR. Para-aminosalicylic acid plasma concentrations in children in comparison with adults after receiving a granular slow-release preparation. J Trop Pediatr. 2013; 59(2):90–94.

54. Kim HJ, Seo KA, Kim HM, Jeong ES, Ghim JL, Lee SH, et al. Simple and accurate quantitative analysis of 20 anti-tuberculosis drugs in human plasma using liquid chromatography-electrospray ionization-tandem mass spectrometry. J Pharm Biomed Anal. 2015; 102:9–16.

55. Hee KH, Seo JJ, Lee LS. Development and validation of liquid chromatography tandem mass spectrometry method for simultaneous quantification of first line tuberculosis drugs and metabolites in human plasma and its application in clinical study. J Pharm Biomed Anal. 2015; 102:253–260.

56. Han M, Jun SH, Lee JH, Park KU, Song J, Song SH. Method for simultaneous analysis of nine second-line anti-tuberculosis drugs using UPLC-MS/MS. J Antimicrob

Chemother. 2013; 68(9):2066–2073.

57. Park SI, Oh J, Jang K, Yoon J, Moon SJ, Park JS, et al. Pharmacokinetics of second-line antituberculosis drugs after multiple administrations in healthy volunteers. Antimicrob Agents Chemother. 2015; 59(8):4429–4435.

58. Lee K, Jun SH, Han M, Song SH, Park JS, Lee JH, et al. Multiplex assay of second-line anti-tuberculosis drugs in dried blood spots using ultra-performance liquid chromatography-tandem mass spectrometry. Ann Lab Med. 2016; 36(5):489–493.

Chapter 3

Aims and Objectives

The aim of this study was to assess the association between adverse gastrointestinal reactions and the concentrations at which, the metabolites of PAS, namely acetyl-PAS (APAS) and glycine-PAS (GPAS) are present in plasma. As part of an ongoing project, the population consisted of adult patients treated for MDR- or XDR-TB who had been dosed with 4g twice-daily and 8g once-daily GSR-PAS on two separate occasions. In an approach to define contributors of toxicity the following objectives were performed:

1. The first objective was to measure the levels of APAS, GPAS and PAS in the population, using ultra high performance liquid chromatography (UHPLC) and mass spectrometry (MS).
2. Once the concentrations were determined, the second objective was to describe the pharmacokinetics (PK) of each compound. With this we were able to determine whether differences exist in the PK of PAS under the two dosing strategies.
3. The third objective statistically evaluated the relationship between peak concentrations of APAS and GPAS and the measure of intolerance, designated as a VAS score, using a Spearman's correlation test.

Chapter Four

Quantification of *para*-aminosalicylic acid (PAS) and metabolites (APAS, GPAS) in human plasma by UHPLC-MS/MS

The current project is a follow up of the original study conducted in 2013 at Clinical Pharmacology (Stellenbosch), whereby a HPLC-MS method was used to quantify the parent drug para-Aminosalicylic acid (PAS). The same population was studied in the current project, with the addition of determining the metabolite (Acetyl-PAS and Glycine-PAS) concentrations. In order to endorse the validation of the assay presented in this chapter, method characteristics such as the sample extraction procedure and post extraction stability of PAS was maintained, as previously described and validated in the original study.

4.1. Introduction

In recent years, several methods have been developed for the simultaneous analysis of second-line anti-tuberculosis (anti-TB) drugs by HPLC and UHPLC, which included PAS.[1–3] The combination of these methods is regarded as the gold standard for identification and quantification analyses of drugs for pharmacokinetic studies in complicated matrices. To the best of our knowledge, assays for the simultaneous screening of PAS and its metabolites in human plasma have not previously been developed. This chapter discusses the application of UHPLC combined with tandem mass spectrometry (MS/MS) using electrospray ionization (ESI) as a tool for the analysis of PAS and its metabolic derivatives acetyl-PAS (APAS) and glycine-PAS (GPAS). We report the method validation according to internationally accepted criteria for bio-analytical drug analysis.

Limited data exist in the literature regarding the pharmacokinetic profile of PAS, APAS and GPAS, when PAS is administered as the granular slow release formulation in patients with MDR or/and XDR-TB. The influence of single and divided dosing, as well as various dosing conditions (i.e. the intake of food, fruit juice, antacids) has been reported for PAS in TB patients

and in healthy volunteers.[4,5] A more recent study by Sy *et al.*[6] investigated the pharmacokinetics of a granular slow release (GSR) formulation of PAS in patients with multi-drug resistant (MDR) or extensively drug resistant (XDR) TB. The pharmacokinetics of PAS was determined in patients receiving two regimens (8g once-daily followed by 4g twice-daily) and the influence of *N*-acetyltransferase 1 (*NAT1*) and *NAT2* genes on PAS concentrations. The influence of *NAT1* and *NAT2* on tolerability was also investigated. Sy and colleagues suggested that the metabolite of PAS, namely APAS and GPAS, are worth investigating to determine the influence of their concentrations on tolerability.[6]

Therefore, the aim of the study was to firstly elicit the pharmacokinetics of APAS, GPAS and parent PAS (see chemical structures in **Figure 4.1**) in plasma samples, using the above described instrumentation. Secondly, the study investigates the influence of metabolite plasma concentrations on the tolerability of GSR-PAS administered orally in an 8g once-daily dose and 4g twice-daily dose.

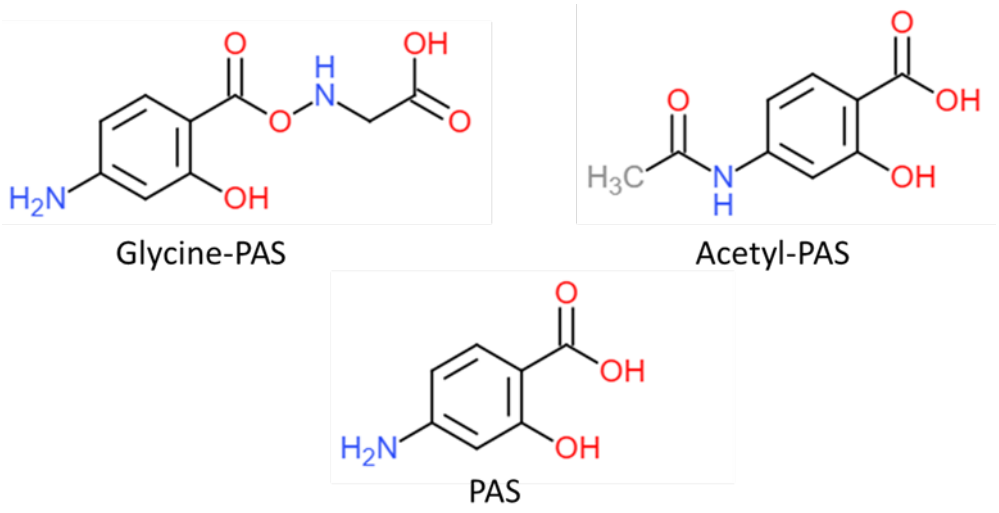

Figure 4.1: Chemical structures of the investigated compounds, glycine-PAS (GPAS, *p*-aminosalicyluric acid), acetyl-PAS (APAS, *N*-acetyl-*p*-aminosalicylic acid) and parent drug PAS (PAS, *p*-aminosalicylic acid).

4.2. Experimental methods

4.2.1 Chemicals and Reagents

All reference standards including PAS, APAS, the internal standard thiacetazone (THIA) and formic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Analytical grade methanol, and acetonitrile were purchased from ROMIL Ltd (Cambridge, England). Water was purified through a Milli-Q water purification system (Millipore, Milford, USA). Drug-free human plasma was obtained from Tygerberg Hospital Blood Bank (Western Cape, South Africa), and heparinized prior to use.

The second metabolite derivative, GPAS was synthesized from the parent PAS. Briefly, the synthesis involved a double protection followed by the acid coupling and global deprotection. The identity of GPAS was confirmed by the Waters Synapt G2 high resolution LC-MS/MS and NMR, included in the supplementary material. The purity of GPAS was found to be more than 99% by HPLC.

4.2.2 Preparation of calibration standards and quality control samples

Stock solutions of PAS, APAS, and GPAS were prepared separately by dissolving 10 mg of each pure powder in 10 mL methanol to achieve a primary concentration of 1 mg/mL. The internal standard, THIA (1 mg/mL) was also prepared in methanol. A working solution of THIA was then prepared in methanol to achieve a final concentration of 2.0 µg/mL. All the stock solutions were kept in volumetric glass flasks and stored at 4 °C.

The plasma standards for calibration curves were prepared by diluting appropriate volumes of PAS, APAS and GPAS standard stock solutions with blank plasma. A series of standards were

prepared to yield final concentrations of 0.52 µg/mL – 167 µg/mL. Quality control samples (QCs) were prepared at 1 µg/mL (limit of low quality control, LLOQ), 4.2 µg/mL (low quality control, LOQ), 17.0 µg/mL (medium quality control, MCQ), and 100.0 µg/mL (high quality control, HQC).

4.2.3 Sample extraction

The frozen plasma samples were thawed at room temperature before the analysis. Aliquots of 250 µL for each plasma sample were dispensed into 2mL microcentrifuge tubes. A volume of 750 µL of internal standard working solution (2.0 µg/mL) was added to each sample, for protein precipitation. The mixture was vortexed for 1min, and centrifuged at 11 000 rpm for 3 min. Eight hundred micro litres (800 µL) of supernatant was transferred to a suitable auto sampler vial and sealed cap, before storage in a refrigerator at 4 ˚C prior to analysis within 1 day. The plasma standard samples were prepared similarly prior to LC-MS/MS analysis.

4.2.4 LC-MS/MS instrumentation and conditions

Liquid chromatography was performed using a Waters Xevo TQS triple quadrupole mass spectrometer connected to a Waters Acquity UPHLC system equipped with a temperature controlled sample organiser. Electrospray ionisation was performed in the positive mode at a desolvation temperature of 350°C, desolvation gas was nitrogen at a flow rate of 800L/h, capillary voltage of 3.8kV and the multiple reaction monitoring (MRM) settings are summarised in **Table 4.1**. Separation was achieved using a reverse-phase Waters HSS T3 column (1.8µm particle size, 2.1 x 50 mm), which was purchased from Waters Corporation (Milford, MA, USA). The method followed a binary gradient mobile phase A containing water

and 0.1% formic acid while mobile phase B contained 0.1% formic acid in acetonitrile. The flow rate was kept at 0.5 mL/min, the gradient started at 95% of A and followed a linear curve to 70% of A over 3 minutes, followed by a wash step for half a minute at 100% of B and re-equilibration to initial conditions to yield a total run time of 4.5 minutes.

Table 4.1: Multiple reaction monitoring (MRM) conditions of each analyte and internal standard.

Analyte	M+H	Retention Time (minutes)	Cone Voltage (V)	Quantifier product ion (Collision energy)	Quantifier product ion (Collision energy)
PAS	154	1.44	15	91 (25)	136 (10)
APAS	196	1.97	15	80 (25)	136 (15)
IS	237	2.17	20	120 (20)	161 (15)
GPAS	211	1.38	15	80 (25)	136 (10)

4.2.5 Method validation

The sample preparation method used in the study, is a protein precipitation extraction protocol that has been developed and validation by Liwa *et al.*[7] For this reason a partial validation of only the newly developed UPHLC-MS/MS method was done and is described below. The freeze and thaw stability of PAS and its metabolite APAS has been assessed and published by Hong *et al.*[8], and adapted to the assay.

4.2.5.1. Selectivity and Specificity

Specificity was studied by testing for lack of interference and the absence of co-eluting peaks in the same MRM channel. Chromatographs of samples from various matrices (blank plasma from three different lots, methanol containing and samples spiked with all analytes) were compared.

4.2.5.2. Accuracy and Precision

The accuracy of the method was determined by 6x injection of QCs at four different concentrations (LLOQ, LOQ, MQC, HQC) within a batch for intra-day accuracy. A QC and blank was included after every 20 injections. The inter-day batch accuracy of the assay was evaluated at the same QC concentrations on three different days. A percentage recovery was recorded as the difference between the mean measured values and experimental value. Precision was recorded as a percentage of relative standard deviation (RSD), and included acceptance criteria for intra and inter-batch as no more than 20% for LLOQ and 15% at higher concentrations.

4.2.5.3. Linearity and LLOQ

Calibration standards were prepared by dilution of PAS, APAS and GPAS stock solution with blank plasma, proceeded with IS. The entire concentration range (0.52 µg/mL – 167 µg/mL) included the LOD. The curve was constructed by plotting the peak area ratio (y-axis) of analytes PAS, APAS and GPAS to the internal standard, versus, the corresponding concentrations (x-axis).

4.2.5.4. Stability

The post preparation stability of PAS, APAS and GPAS in plasma was maintained by storage in a refrigerator at 4º C prior to LCMS analysis within 24 hours.[8] The long term storage stability (-80º C) of the clinical samples was determined by comparing the concentrations of PAS in the original study[9] with those achieved in the presented study. The percentage recovery of PAS was determined and an RSD of 15% was acceptable.

4.2.6 Clinical application

As part of an on-going project, the study protocol was approved by the Health Research Ethics Committee at Stellenbosch University (S17/05/107), and followed the guidelines of the Helsinki Declaration of 1975 and its amendments.

4.2.6.1 Study subjects and design

The two-week crossover study was conducted in 29 adult MDR and XDR-TB patients hospitalised at the Brooklyn Chest Hospital, Cape Town. All subjects participated in the PK study, which involved one week (Day 1 – 8) of 8 g once-daily PAS dosing followed by one week (Day 9 – 16) of 4 g twice-daily dosing. Blood samples were collected in EDTA-containing tubes at 1, 2, 3, 4, 6, 8, 12 and 24 hours (post-dose) on days 8 and 16, with a 10-minute deviation time. The specimens were collected on ice, centrifuged for 10 minutes at 3500 rpm, where after the plasma was harvested and stored at -80˚ C until analysis. The plasma samples were spiked with the internal standard and treated as per the extraction procedure described above. Dual analysis of the clinical samples and QC samples was performed.

4.2.6.2 Pharmacokinetic analysis

The PK parameters of PAS, APAS and GPAS, were determined by non-compartmental analysis (NCA) using Winnonlin (Princeton, NJ, USA) version 8.0. C_{max} and time to C_{max} (T_{max}) were determined from the plasma concentration time data. The software default linear trapezoidal method was used to assess the area under the curve from zero up to 24 hour concentration (AUC_{0-24}).

4.2.6.3 Tolerability and safety

Tolerance of PAS was determined using a visual analogue scale (VAS) rating gastrointestinal (GI) adverse effects (AEs) including nausea, vomiting, abdominal pain and cramps, diarrhoea, and bloating. The form contained 10 vertical demarcations, separated by 1 cm each, with the most left indicating no symptoms and the most right indicating severe symptoms. The subjects were asked to complete an evaluation on a daily basis after dosing, by circling the mark that most closely fitted their perception of the symptom.

4.2.6.4 Statistical analysis

Frequency and percentage were determined for subject demographic characteristics (gender, race, TB diagnosis, HIV status). The median as well as interquartile range (IQR) value which is reported as the 75th and 25th percentile, were calculated for all measured data including subject characteristics (age, height, body weight and body mass index), PK parameters of each compound (C_{max}, C_{min}, AUC_{0-24}, and T_{max}), and gastrointestinal AEs (nausea, vomiting, bloating, diarrhoea, and abdominal pain and cramps), using STATA statistical software (Texas, USA) version 15.1.

All measured data were evaluated for normality using the Shapiro-Wilk test. Data not normally distributed were evaluated using non-parametric tests.

PK parameters of each compound were compared for any differences using a non-parametric Wilcoxon signed-rank test (STATA), on the two dosing regimens. The same was done for the VAS scores of each adverse effect between the two doses.

Correlation analysis (STATA) was performed to measure the association between each adverse effect and the median C_{max} (PK parameter) of PAS, APAS and GPAS using a non-parametric Spearman's rank correlation coefficient (Spearman's Rho). Statistical significance was set at a *P* value less than 0.05.

4.3. Results

4.3.1 MS/MS detection optimization

Optimization of the mass spectrometry conditions was performed to achieve the maximum stable response of the precursor ions and corresponding product ions of the analytes in question. The tuning of MS parameters was performed in positive and negative ionization modes for PAS, APAS, GPAS and internal standard THIA. Both positive and negative modes showed signal for all analytes. The positive mode displayed a higher response and was therefore selected. The Q1 MS full scan mass spectra of PAS, APAS and GPAS was dominated by protonated precursor ions $[M+H]^+$ at *m/z* 154, 196 and 211 respectively. These precursor ions were used to obtain the Q3 product ion spectra. Hereby, the mass transition ion pair was selected as *m/z* 154>91; 154>119; 154>136 for PAS, and *m/z* 196>80; 196>136; 196>178 for APAS, and *m/z* 211>80; 211>108; 211>136 for GPAS, due to the high similarity in structure between the analytes. The inherent selectivity of MS/MS detection was expected to be beneficial in developing a selective and sensitive method. The MRM parameters such as cone voltage and collision energy were optimized to obtain a constant and suitable intensity for all analytes.

4.3.2 LC separation optimization

The composition of the mobile phase and column was optimized to achieve adequate chromatographic resolution and symmetrical peak shape within a short run time. Various combinations of solvents such as methanol and acetonitrile along with altered ratios of formic acid were evaluated on Waters BEH C18, Xbridge C18, Agilent Zorbax C18 and Waters HSS T3 C18 columns. The mobile phase consisting of 0.1 % formic acid in acetonitrile[7,10] mixture

(minute, % mobile phase B): 0.30, 5; 3.00, 30; 3.10, 100; 3.60, 100; 3.80, 5 delivered on the Waters HSS T3 C18 column (1.8 µm, 2.1 x 50 mm)[1,3] at a flow rate of 0.5 mL/min was found to be most suitable for faster elution, better reproducibility and peak shape for all targeted analytes.

4.3.3 Selection of internal standard

A number of compounds were evaluated to find an appropriate internal standard. Based on the compound's chromatographic behaviour and extraction efficiency, thiacetazone was found to be the most appropriate IS comparable with the targeted analytes.

4.3.4 Selectivity and specificity

The representative chromatographs obtained from various matrices, including blank plasma spiked with LLOQ 0.5 standard of each analyte and IS are presented in **Figure 4.2**. The chromatographs expressed no significant endogenous interferences observed in the respective MRM channel at the retention time of PAS (1.44 min), APAS (1.97 min), GPAS (1.38 min) and THIA (2.17 min). The short run time of 4.5 min, showed the method to be suitable for routine analysis.

4.3.5 Accuracy and precision

Intra- and inter-day accuracy and precision are presented in **Table 4.2** for each PAS, APAS and GPAS. The inter-batch precision (%RSD) at LLOQ, LQC, MQC and HQC levels varied from 4.7% to 8.3% for PAS, 2.23% to 10.2% for APAS and 3.1% to 13.0% for GPAS. The intra-batch varied from 2.9% to 9.2% for PAS, 0.9% to 7.9% for APAS and 1.8% to 4.8% for GPAS.

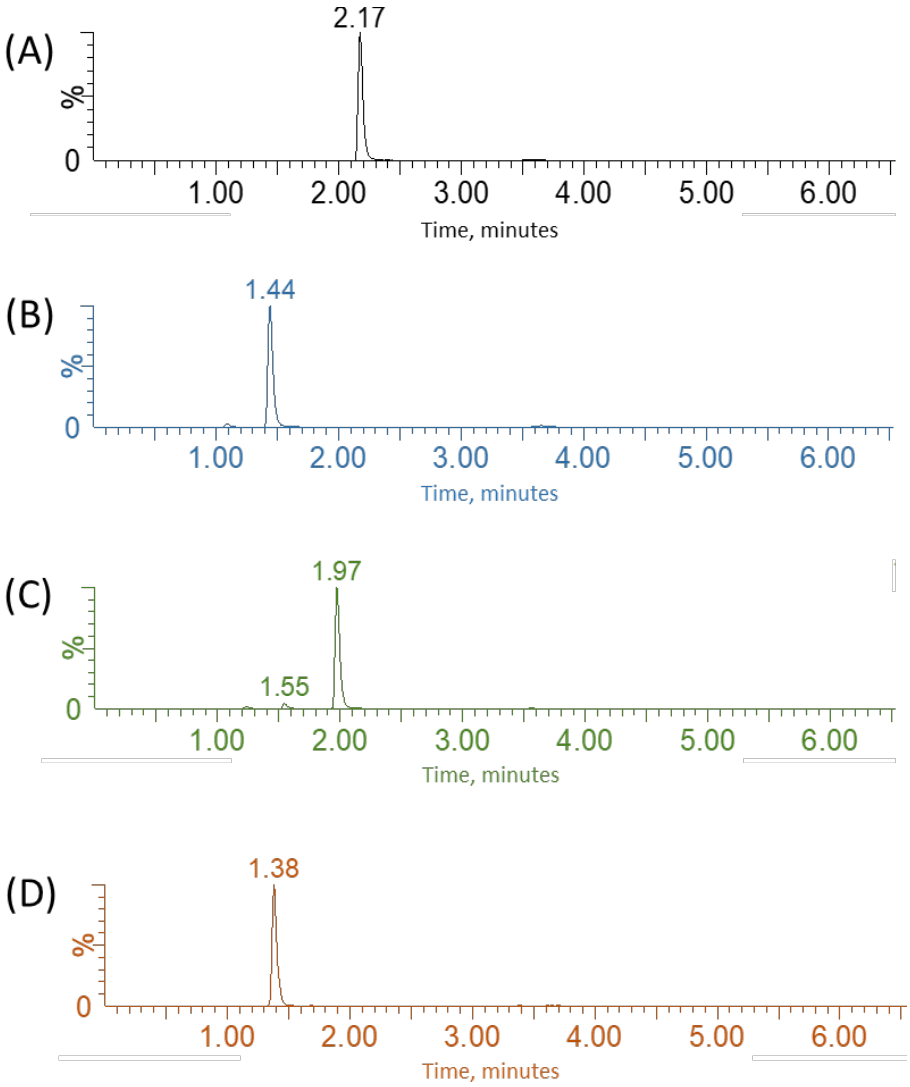

Figure 4.2: Ion chromatograms for (A) internal standard THIA, (B) PAS, (C) APAS, and (D) GPAS, achieved by blank plasma spiked with 0.500 µg/mL of each standard. The retention times of IS, PAS, APAS and GPAS are 2.17 min, 1.44 min, 1.97 min, and 1.38 min respectively.

4.3.6 Linearity and detection range

The nine-point calibration curve was linear over the concentration range of 0.52 – 167 µg/mL for all targeted analytes, with correlation coefficient in the range of $0.996 \leq r^2 \leq 0.999$ for all calibration curves on all 4 days of analysis. **Table 4.3** summarizes the calibration curve results for each analyte. The accuracy results varied from 96.50% to 114.00% while the precision (%RSD) results varied from 0.20% to 6.40% amongst all analytes. Due to the very high concentrations that the analytes are present in the plasma, the limit of quantification was taken as 120% above the reading of the blank injected after the highest plasma standard.

Table 4.2: Intra- and inter-batch precision and accuracy of PAS, APAS and GPAS (mean µg/mL, RSD %, accuracy %).

Analyte	Theoretical Concentration (µg/mL)		Intra-day (n=5)			Inter-day (n=6)		
			Mean (µg/mL)	RSD (%)	Accuracy (%)	Mean (µg/mL)	RSD (%)	Accuracy (%)
PAS								
	LLOQ	1	1.01	9.2	103.3	1.05	7.9	105.3
	LQC	4.2	4.19	2.9	99.8	4.05	8.3	96.5
	MQC	17	16.78	3.6	98.7	17.41	7.2	102.4
	HQC	100	100.14	4.8	100.1	97.44	4.7	97.4
APAS								
	LLOQ	1	1.11	6.0	111.0	1.11	5.9	110.9
	LQC	4.2	4.18	1.4	99.6	4.23	2.3	100.7
	MQC	17	16.90	0.9	99.4	16.85	5.8	99.14
	HQC	100	105.71	7.9	105.7	106.34	10.2	106.3
GPAS								
	LLOQ	1	1.09	3.7	109.2	1.14	13	114
	LQC	4.2	4.19	1.8	99.8	4.13	3.1	98.4
	MQC	17	17.24	2.6	101.4	17.21	3.2	101.2
	HQC	100	94.63	4.8	94.6	96.13	4.4	96.1

LLOQ = lower limit of quantification; **LQC** = low quality control; **MQC** = medium quality control; **HQC** = high quality control.

Table 4.3: Linearity of calibration standards (mean µg/mL, RSD %, accuracy %).

µg/mL	PAS (n=6)			APAS (n=6)			GPAS (n=6)		
	Mean (µg/mL)	RSD (%)	Accuracy (%)	Mean (µg/mL)	RSD (%)	Accuracy (%)	Mean (µg/mL)	RSD (%)	Accuracy (%)
0.52	0.59	6.4	114.0	0.51	9.2	97.9	0.56	4.1	108.5
1.5	1.53	1.8	101.9	1.51	1.4	100.9	1.51	3.3	100.5
2.1	2.10	4.3	99.9	2.33	4.2	110.7	2.19	3.7	104.1
4.2	4.21	1.8	100.3	4.22	1.4	100.6	4.21	1.3	100.3
8.3	8.78	1.3	105.8	8.59	4.1	103.5	8.48	1.3	102.2
17	17.09	0.8	100.5	17.37	4.2	102.2	17.05	2.2	100.3
33	33.65	0.6	100.8	33.13	1.2	99.2	33.16	2.2	99.3
66	65.06	1.2	98.6	65.25	0.2	98.9	63.72	7.3	96.5
167	167.10	1.0	100.1	166.41	1.0	99.6	166.44	1.2	99.7

4.3.7 Study subjects

Subjects' characteristics and concomitant medications administered are summarized in **Tables 4.4, 4.5 and 4.6**. Twenty-five (n=25) subjects completed both dosing regimens while four (n=4) subjects had PK data for either the once-daily dosing (n=2) or the twice-daily dosing regimen (n=2), but not for both. Statistical analysis was performed on all 29 subjects in the study.

Twenty-five subjects (86%) were Coloured (mixed racial decent), four subjects (14%) African Blacks, and there were no White or Asian study participants. Seventeen subjects (59%) were male. Five males (17%) were HIV positive, while 6 (21%) males had XDR-TB. The mean age

in males was 35 years. Twelve subjects (41%) were female. Three females (10%) were HIV positive, and one (3%) had XDR-TB. The mean age among females was 38 years.

Subjects showed no difference between the two dosing regimen periods based on body weight and body mass index (BMI).

Table 4.4: Demographic characteristics of subjects crossed over in the study.

Characteristic	**Subjects (n=29)**
Gender (%)	
Male	17 (59%)
Female	12 (41%)
Race (%)	
Coloured	25 (86%)
Black	4 (14%)
TB diagnosis (%)	
MDR	22 (76%)
XDR	7 (24%)
HIV Status (frequency, %)	
Negative	21 (72%)
Positive	8 (28%)
Age (years)	
Median (IQR)	34 (29 – 44)
Height (m)	
Median (IQR)	1.7 (1.6 – 1.7)

IQR = interquartile range; **Coloured** = is the term applied to racially distinct group of individuals with mixed ancestry, residing in South Africa.(11)

Table 4.5: A comparison of body weight and BMI as recorded at the start of each regimen throughout the cross over.

Characteristic	Dosing regimens of PAS		
	8 g once-daily	4 g twice-daily	P-value
Body weight (kg)			
Median	56.5	58.5	0.2065
IQR	50 – 62.3	50 – 62.0	
Body Mass Index (kg/m^2)			
Median	20.1	20.3	0.0728
Range	15.1 – 25.2	16.0 – 34.7	

IQR = interquartile range.

Table 4.6: Summary of all concomitant medication administered with PAS.

Drug category	Subjects (n) Frequency (%)
Anti-tuberculosis	
Capreomycin	24 (83%)
Moxifloxacin	27 (93%)
Ethambutol	25 (86%)
Ethionamide	24 (82%)
Isoniazid	12 (41%)
Kanamycin	2 (7%)
Teridizone	26 (90%)
Pyrazinamide	26 (90%)
Clarithromycin	1 (3%)
Clofazimine	9 (31%)
Antiretroviral	
Efavirenz	6 (21%)
Stavudine	5 (17%)
Lamivudine	6 (21%)
Gastrointestinal medicines	
Metoclopromide	6 (21%)
Ranitidine	4 (14%)
Vitamins	
Pyridoxine	27 (93%)
Vitamin B Complex	11 (38%)
Antidepressants and Psychotics	
Amitriptyline	5 (17%)

4.3.8 Pharmacokinetics of PAS and metabolites

Data was available for 27 patients on the once-daily regimen and 25 patients on the twice- daily regimen, respectively, **Table 4.7**. T_{max} of APAS was statistically significantly different between the two regimens (p=0.0144). This was the same for C_{max} (p=0.0006), AUC_{0-24} (p=0.0009), and T_{max} (p=0.0189) in parent drug PAS.

Table 4.7: Summary of the pharmacokinetic parameters of PAS, APAS and GPAS at both dosing regimens.

PK Parameter	Dosing regimen		P-value
	8 g once-daily (n=27)	4 g twice-daily (n=25)	
PAS (median, IQR)			
C_{max} (mg/L)	82.69 (62.43 – 102.41)	53.63 (40.42 – 68.55)	0.0006*
AUC_{0-24} (mg.h/L)	950.93 (610.84 – 1274.97)	609.55 (367.07 – 821.40)	0.0009*
T_{max} (h)	8 (4 – 8)	6 (3 – 8)	0.0189*
APAS (median, IQR)			
C_{max} (mg/L)	16.36 (11.31 – 20.30)	14.59 (10.00 – 22.36)	0.6500
AUC_{0-24} (mg.h/L)	239.08 (185.36 – 376.07)	235.28 (177.61 – 326.00)	0.3065
T_{max} (h)	8 (6 - 8)	6 (4 - 8)	0.0144*
GPAS (median, IQR)			
C_{max} (mg/L)	6.08 (4.56 – 9.45)	5.02 (3.90 – 8.02)	0.2130
AUC_{0-24} (mg.h/L)	74.66 (41.38 – 106.47)	63.45 (44.95 – 83.31)	0.0575
T_{max} (h)	8 (4 – 8)	4 (3 – 8)	0.5233

IQR = interquartile range; * = indicates statistical significant P value < 0.05

The plasma concentration time profile for each compound (PAS, APAS and GPAS) is presented at each dosing regimen (4 g twice-daily and 8 g once-daily at steady state conditions) in **Figure 4.3**. The 8 g once-daily regimen parent PAS and its metabolites APAS and GPAS had a higher peak concentration (C_{max}) compared to the 4 g twice-daily regimen whilst the peak concentrations were reached earlier in the 4 g twice-daily regimen (**Table 4.7**).

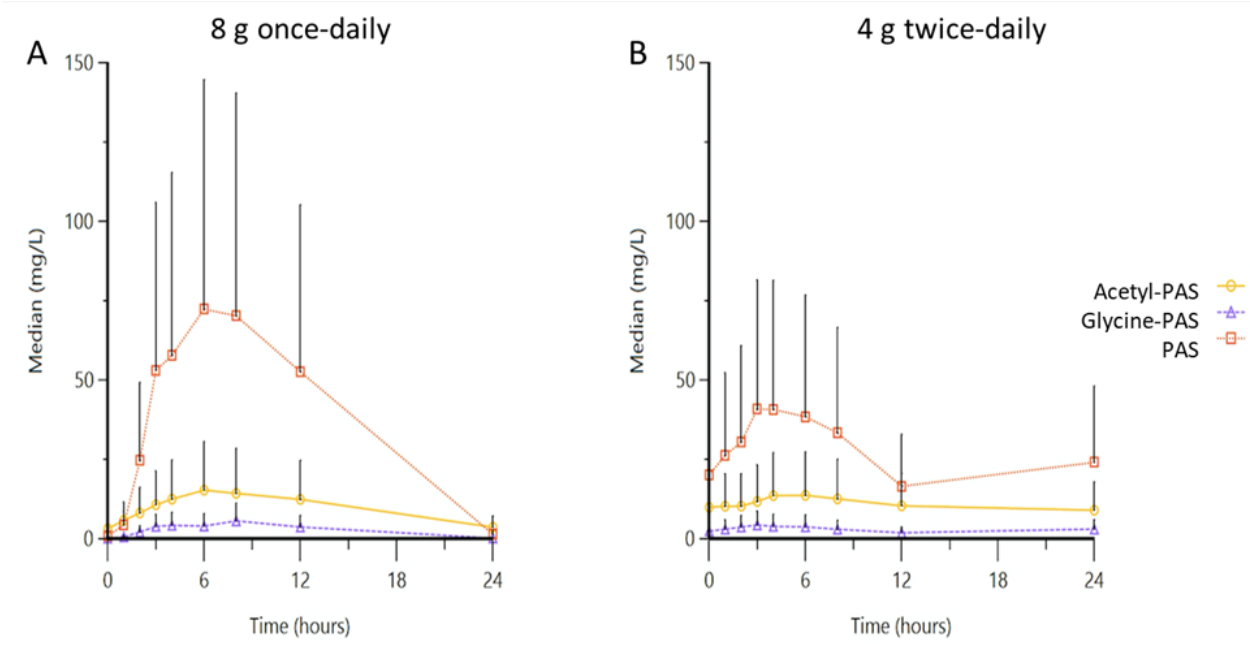

Figure 4.3: Plasma concentrations (mg/L) for PAS (orange), APAS (yellow) and GPAS (purple) in subjects receiving 8 g once-daily regimen (A) and 4 g twice-daily dosing regimen (B), over 24 hours. Each time point presents the median concentration and vertical lines represent the interquartile range around the point.

4.3.9 Tolerability and safety

VAS score data was available for all subjects. **Table 4.8** summarises the median score for each AE and for both regimens. The Wilcoxon sign-rank test reported statistically significant more abdominal pain and cramps in the twice-daily regimen compared with once-daily (p=0.018) regimen, while there was no statistically significant difference noted in any other category of GI adverse effects.

Table 4.8: Summary of gastrointestinal adverse effects (nausea, vomiting, bloating, diarrhoea, abdominal pain and cramps) at both dosing regimens.

Adverse Effect	Dosing regimen of PAS		P-value
	8 g once-daily (Median; IQR)	4 g twice-daily (Median; IQR)	
Nausea	0.37 (0.05 – 1.66)	0.44 (0.03 – 1.39)	0.899
Vomiting	0 (0 – 0.13)	0 (0 – 0.25)	0.541
Bloating	0 (0 – 0.65)	0.15 (0 – 0.62)	0.311
Diarrhoea	0.44 (0 – 2.01)	0.31 (0 – 1.59)	0.119
Abdominal pain and cramps	0 (0 – 0.08)	0.14 (0 – 0.59)	0.018*

* = significant P-value < 0.05

4.3.10 Relationship between peak concentrations and gastrointestinal AE's

Displayed in **Tables 4.9, 4.10** and **4.11** is the Spearman's correlation coefficient and its corresponding p-value, which measured the association between each adverse effect and the median C_{max} of PAS, APAS, and GPAS, respectively. Statistically significant correlations were observed for APAS concentration in terms of bloating (p= 0.025) and diarrhoea (p=0.044), respectively. Similarly, GPAS concentration showed a significant correlation with diarrhoea (p= 0.041). Although significant correlations are seen, the strength of the associations as described by the Spearman's rho are all below 0.05 and thus represents a very low correlation as seen in **Figure 4.4** and **4.5**. The negative rho values indicate that with increasing concentrations of APAS and GPAS, less bloating and diarrhoea is observed.

Table 4.9: Correlation between the median AE score and median plasma concentrations of parent compound PAS, at each dosing regimen.

Adverse effect	8 g once-daily Spearman's Rho (P-value)	4 g twice-daily Spearman's Rho (P-value)
Nausea	0.151 (0.461)	0.089 (0.673)
Vomiting	0.048 (0.817)	0.099 (0.640)
Bloating	0.204 (0.318)	0.123 (0.558)
Diarrhoea	0.024 (0.908)	-0.384 (0.058)
Abdominal pain and cramps	0.027 (0.897)	-0.153 (0.466)

* = significant; P-value < 0.05

Table 4.10: Correlation between the median AE score and median plasma concentrations of metabolite APAS, at each dosing regimen.

Adverse effect	8 g once-daily Spearman's Rho (P-value)	4 g twice-daily Spearman's Rho (P-value)
Nausea	-0.189 (0.355)	-0.189 (0.365)
Vomiting	0.079 (0.698)	0.062 (0.769)
Bloating	-0.074 (0.719)	-0.448 (0.025)*
Diarrhoea	-0.337 (0.092)	-0.407 (0.044)*
Abdominal pain and cramps	-0.042 (0.839)	-0.327 (0.111)

* = significant; P-value < 0.05

Table 4.11: Correlation between the median AE score and median plasma concentrations of metabolite GPAS, at each dosing regimen.

Adverse effect	8 g once-daily Spearman's Rho (P-value)	4 g twice-daily Spearman's Rho (P-value)
Nausea	0.140 (0.496)	-0.378 (0.062)
Vomiting	0.016 (0.937)	-0.177 (0.398)
Bloating	0.195 (0.3398)	0.023 (0.912)
Diarrhoea	-0.319 (0.112)	-0.412 (0.041)*
Abdominal pain and cramps	0.053 (0.798)	-0.108 (0.609)

* = significant; P-value < 0.05

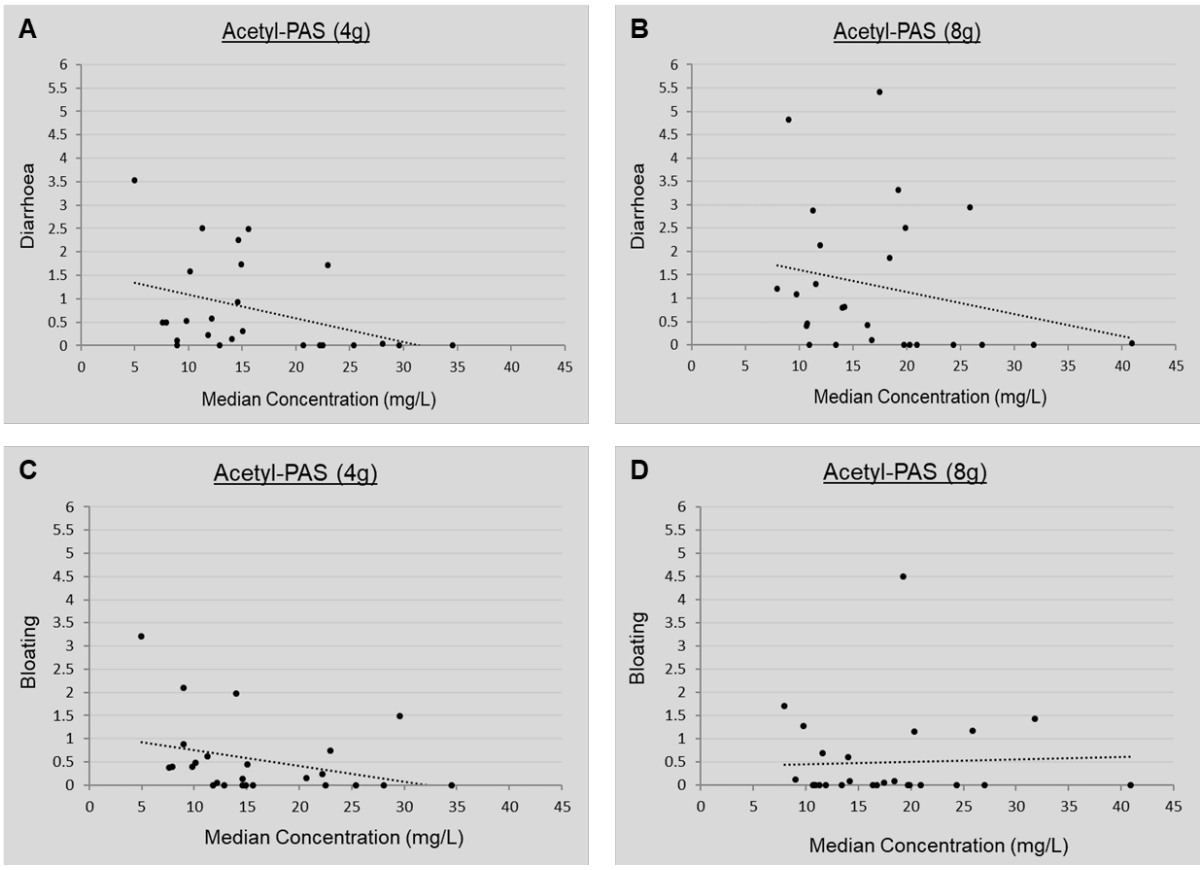

Figure 4.4: Scatter plot of the median plasma maximum concentration of APAS versus the VAS score for adverse effects, on each dosing regimen. Significant correlations were observed for APAS and bloating on 4g twice-daily regimen, p= 0.025; rho= -0.448 (C) and APAS and diarrhoea, p = 0.044; rho= -0.407 (A).

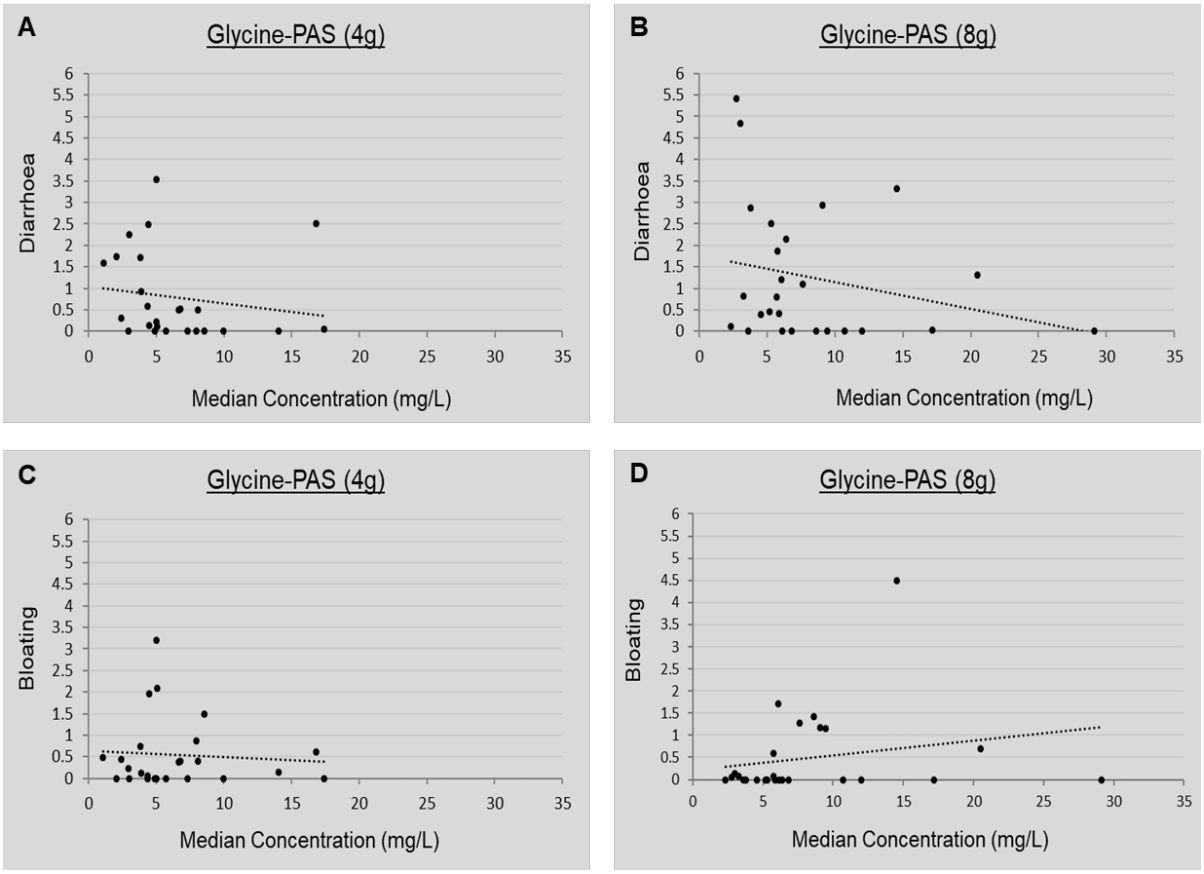

Figure 4.5: Scatter plot of the median plasma maximum concentration of GPAS versus the VAS score for adverse effects, on each dosing regimen. One significant correlation was observed for GPAS and diarrhoea on 4g twice-daily regimen, p = 0.041; rho= -0.412 (A).

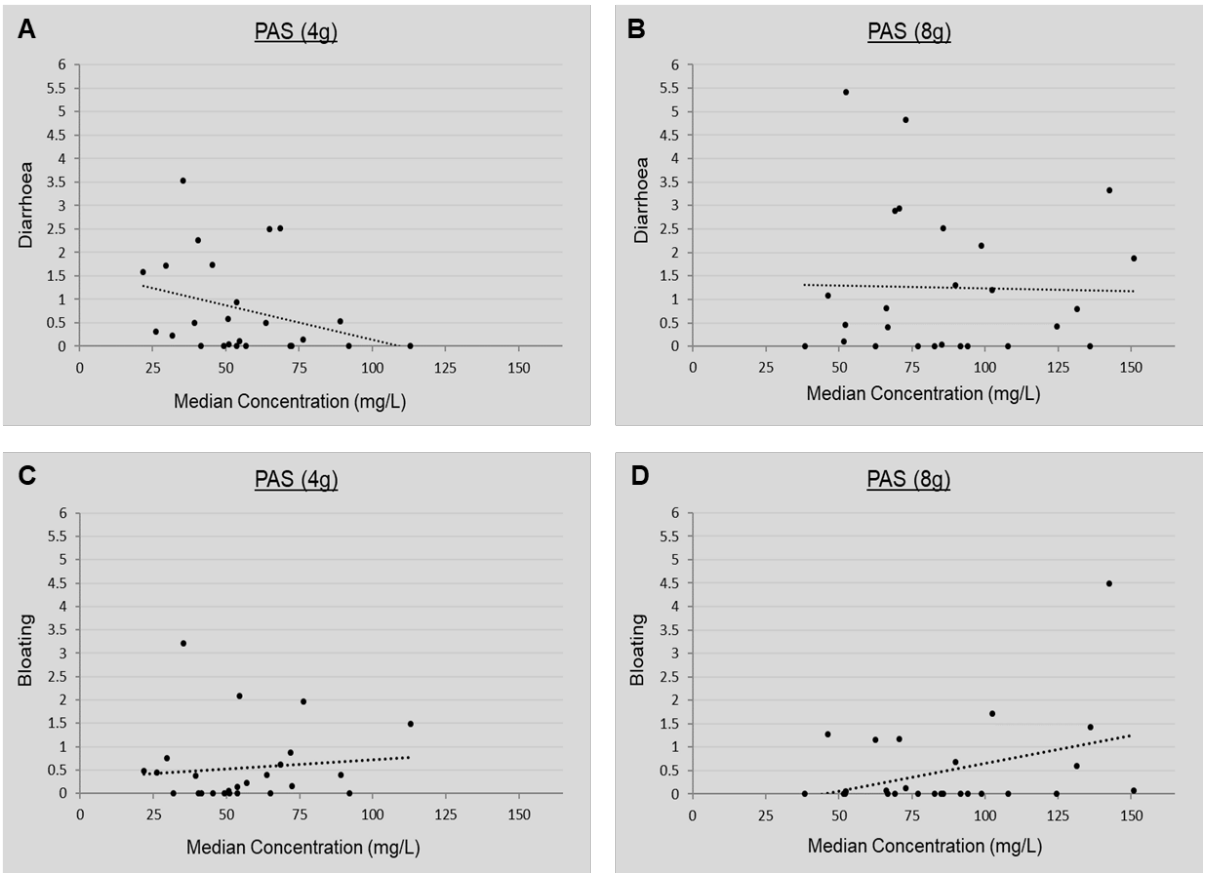

Figure 4.6: Scatter plot of the median plasma maximum concentration of PAS versus the VAS score for adverse effects, on each dosing regimen. No significant correlations were observed between these two variables.

References

1. Han M, Jun SH, Lee JH, Park KU, Song J, Song SH. Method for simultaneous analysis of nine second-line anti-tuberculosis drugs using UPLC-MS/MS. J Antimicrob Chemother. 2013; 68(9):2066–2073.

2. Park SI, Oh J, Jang K, Yoon J, Moon SJ, Park JS, et al. Pharmacokinetics of second-line antituberculosis drugs after multiple administrations in healthy volunteers. Antimicrob Agents Chemother. 2015; 59(8):4429–4435.

3. Lee K, Jun SH, Han M, Song SH, Park JS, Lee JH, et al. Multiplex assay of second-line anti-tuberculosis drugs in dried blood spots using ultra-performance liquid chromatography-tandem mass spectrometry. Ann Lab Med. 2016; 36(5):489–493.

4. Peloquin CA, Berning SE, Huitt GA, Childs JM, Singleton MD, James GT. Once-daily and twice-daily dosing of p-aminosalicylic acid granules. Am J Respir Crit Care Med. 1999; 159(3):932–934.

5. Peloquin CA, Henshaw TL, Huitt GA, Berning SE, Nitta AT, James GT. Pharmacokinetic evaluation of para-aminosalicylic acid granules. Pharmacother J Hum Pharmacol Drug Ther. 1994; 14(1):40–46.

6. Sy SKB, De Kock L, Diacon AH, Werely CJ, Xia H, Rosenkranz B, et al. N-acetyltransferase genotypes and the pharmacokinetics and tolerability of para-aminosalicylic acid in patients with drug-resistant pulmonary tuberculosis. Antimicrob Agents Chemother. 2015; 59(7):4129–4138.

7. Liwa AC, Schaaf HS, Rosenkranz B, Seifart HI, Diacon AH, Donald PR. Para-aminosalicylic acid plasma concentrations in children in comparison with adults after receiving a granular slow-release preparation. J Trop Pediatr. 2013; 59(2):90–94.

8. Hong L, Jiang W, Zheng W, Zeng S. HPLC analysis of para-aminosalicylic acid and its metabolite in plasma, cerebrospinal fluid and brain tissues. J Pharm Biomed Anal. 2011; 54(5):1101–1109.

9. De Kock L. Pharmacokinetic study of twice-daily versus once-daily dosing with granular slow release para-aminosalicylic acid in adults on second-line anti-tuberculosis and antiretroviral treatment. MSc thesis, Stellenbosch University. 2013.

10. Kim H-J, Seo K-A, Kim H-M, Jeong E-S, Ghim JL, Lee SH, et al. Simple and accurate quantitative analysis of 20 anti-tuberculosis drugs in human plasma using liquid chromatography–electrospray ionization–tandem mass spectrometry. J Pharm Biomed Anal. 2015; 102:19–16.

11. Posel D. What's in a name? Racial categorisations under apartheid and their afterlife. Transformation Durban. 2001; 50–74.

Chapter Five

Discussion, conclusion, limitations and future work

5.1. Discussion

There has only been one previously reported study, which analysed PAS and its major metabolites in blood and urine, using a paper chromatography method[1] and there has to our knowledge not been any published report on the simultaneous determination of PAS, APAS and GPAS in plasma matrix by UHPLC-MS/MS. The development of such a method was thus aimed at extending the quantification accuracy for these compounds while also generating pharmacokinetic data for the anti-TB agent PAS and its metabolites APAS and GPAS. We achieved this by optimizing the chromatographic and mass spectrometric conditions suitable to these three compounds by using a simple and efficient one step protein precipitation extraction procedure. The present study showed a large inter-individual variability between parent drug PAS and its metabolites with regards to concentration time curves, with C_{max} ranging from 40.42 to 102.41 mg/L for PAS. Metabolites ranged between 10.00 to 22.36 mg/L, and 3.90 to 9.45 mg/L for APAS and for GPAS, respectively necessitating a wide linear concentration range of the calibration curve (0.50 – 160.0 mg/L).

PAS is currently used in the treatment of drug-resistant tuberculosis. Since its discovery it has shown substantial efficacy and the ability to prevent acquired resistant in its companion drugs.[2–4] PAS has also displayed the potential to have early bactericidal activity, a result observed when it was administered as a single 15g daily dose[5] and its efficacy was measured by the fall in the CFU of *M. tuberculosis* in sputum samples. Over the first two days, a fall of 0.259 $\log_{10}$ CFU/mL sputum was recorded indicating that although PAS is mainly valued for preventing drug resistance in companion drugs, it also has the ability of bactericidal activity similar to that of first-line drugs, rifampicin and isoniazid.[6,7] At present, PAS is used with reluctance, due to its notoriously gastrointestinal intolerance. Among a large number of studies conducted by the British Medical Research Council[3,4,8] it was concluded that PAS intolerance was undoubtedly affected by the dose.[9–11] Investigations comparing divided daily dosing with

single daily dosing found that intolerance was experienced similarly and probably somewhat better with a single dose than with divided dosing.[9–14] Sy and colleagues[14] reported that neither C_{max} nor AUC_{0-12} were associated with intolerance. However, in terms of other pharmacokinetic parameters, a higher degree of abdominal pain and discomfort and diarrhoea was associated with lower minimum concentration (C_{min}) of PAS.[14] At minimum PAS concentrations, it is expected that concentrations of metabolites, APAS and GPAS are high, due to biotransformation processes. This suggestion has set the premise for the presented work.[14]

In the present study the slow release formulation of PAS was well tolerated, with even distribution of AEs scored across the two dosing regimens. Three subjects (n=3) experienced no gastrointestinal AEs. The remaining 26 had evidence of gastrointestinal AEs, however, the majority of the scores were clustered around zero. The most frequently reported AEs were nausea and diarrhoea, consistently recorded at both dosing regimens. It was noted that abdominal pain and cramps (APC) were statistically more frequent in the 4g twice-daily than the 8g once-daily dose [0.14(0 – 0.59) versus 0(0 – 0.08); median (IQR); p= 0.018], and GI AEs were also not worse than with the higher 8g dose. This finding is in keeping with previously reported studies, by Riska[9–11] and Yue [12], and would suggest that the twice-daily dosing strategy of PAS should be discontinued and replaced by a once-daily higher dose.

In addition the present study looked specifically at the role that the metabolites APAS and GPAS might play in GI adverse events. The Spearman's correlation test was utilised to assess the relationship between AE scores and maximum plasma concentrations (C_{max}) of each metabolite, APAS and GPAS, after oral administration of PAS. There were statistically significant inverse associations between APAS concentrations and bloating (rho= -0.448; p= 0.025) and diarrhoea (rho= -0.407; p= 0.044), respectively, for the twice-daily dose. The same

was found for GPAS concentrations and diarrhoea occurrences (rho= -0.412; p= 0.041). Although this was statistically significant the strength of the correlations, which is described by the rho value (**Table 4.9**), - suggested a relatively low association, and therefore a low linear relationship between metabolite median C_{max} and AEs. Interestingly, the association was an inverse correlation, indicating that as the metabolites APAS and GPAS concentrations increased in the 4 g twice-daily regimen, the occurrence of bloating and diarrhoea decreased (**Figure 4.4 and 4.5**). A possible explanation for this type of relationship is that the main factor of PAS intolerance is its presence in the GIT. Therefore, the presence of high concentrations of metabolites APAS and GPAS would indicate the removal of PAS from the GIT, allowing less intolerance to be experienced. Amongst the correlations, which were not statistically significant, positive relationships between AEs and concentrations of APAS and GPAS were noted.

Our study describes the pharmacokinetics of APAS and GPAS following administration of GSR-PAS and the pharmacokinetic parameter estimates (C_{max}, T_{max}, and AUC_{0-24}) of APAS and GPAS were very similar in both dosing regimens. Importantly we noted that the PAS concentrations remained higher than the conjugates at both doses (4g and 8g), over the 24 hour study period. In earlier reports, Lehmann[1] recounted that in plasma, GPAS concentrations followed the trend of the PAS curve, but in lower quantities. By contrast, APAS conjugation showed to dominate at low doses, such as 4g PAS and then diminish when the dose was increased to 8g PAS. These trends in the concentration time curve were reported after single and repeated oral doses of PAS tablets (4g, 8g, and 12g). Lehmann[1] reported this in plasma as well, whereas Peloquin *et al*.[15] displayed mean serum APAS concentrations exceeding PAS, in healthy volunteers after a single 4g granular PAS dose. In the present study, APAS and GPAS concentrations remained 3-8 fold and 5-10 fold lower that the PAS concentration suggesting that PAS, administered as granular slow release formulation is not so rapidly metabolized due

to the substantially prolonged release and absorption of the granular slow release formulation.[15] Typically, approximately 80% of absorbed PAS is excreted renally, while the remaining 20% is metabolized mainly to APAS and GPAS among other inactive compounds.[16,17] Interestingly, both acetylation and glycine conjugation are saturable processes.[18] It is hypothesized that these processes compete for available acetyl-CoA, a known common substrate.[1,18,19] Very little is known about the enzymes responsible for conjugation of PAS with glycine to form *p*-aminosalicyluric acid (GPAS), while the acetylating enzyme system has been widely studied. Arylamine *N*-acetyltransferases are most importantly involved in the metabolism of PAS, producing APAS which is modulated by *N*-acetyltransferase 1 (*NAT1*) and to a lesser degree *NAT2*.[7,20]

Regarding the pharmacokinetics of PAS, comparison of the median C_{max}, AUC_{0-24} and T_{max} parameters between the 4g twice-daily and 8g once-daily PAS displayed linearity relative to the dosage. This current study noted that PAS was present in similar concentrations, although slightly higher than previously reported.[15,21,22] This may be due to subject variability in our study, as the coefficient of variation for C_{max} in PAS (**Table 4.7**) varied by 34.9% and 39.4% in the 8g dose and 4g dose respectively. Factors such as low body weight, TB disease state, co-infection with HIV or the effects of other concomitant medications may also contribute to these outcomes. Additionally, this particular formulation of PAS provides a slower release while in the stomach and therefore inhibits the rapid acetylation via first pass metabolism, in turn allowing PAS concentrations to be higher.

5.2. Conclusions

The study's research aim and objectives have been addressed in Chapter 3. The findings of the presented work has shown that it is possible to assay PAS and its two major metabolites, APAS and GPAS, in plasma by UHPLC-MS/MS using a single protein precipitation extraction method. This method proves to be simple and effective in routine clinical drug monitoring of patients treated with PAS.

Additionally, our study provides data on the pharmacokinetics of PAS and its metabolites APAS and GPAS, in a population of adult patients with MDR- and/or XDR-TB. A large amount of inter-individual variation was found in the population for the Cmax of the metabolites and parent drug. The granular slow release PAS formulation administered, and the disease state of the population may be contributing factors to this variance. Our data shows that PAS was not as rapidly metabolized as previously reported. This is consistent with the slow release formulation used, which prolonged the absorption and allowed the metabolites to be present in concentrations lower than PAS, over the course of the study period.

By characterizing the PK profiles of PAS and its metabolites we were able to use this data to make inferences regarding the toxic effects of the drug. Opinions may differ to the use of PAS in the treatment of TB. This is often due to the low tolerance of the drug. However, the experimental and clinical findings presented suggest that maximum PAS concentrations achieved from a single 8g daily dose does not pose a higher risk of GIT intolerance. In contrast, more occasions of AE's were experienced in the fractionated regimen. Therefore, a higher single daily dose of PAS is recommended for use in TB regimens as this may be advantageous in preventing acquired resistance in companion drugs as well as improved clinical efficacy of PAS.

5.3. Limitations

The study is limited by the low number of patients studied. This consequently limits the impact of our conclusions, including the statistically significant differences found in the pharmacokinetic parameters described and the type of relationship seen between the metabolite concentrations and occurrence of GIT intolerance. Therefore, a further study of PAS and its metabolites and their influence on toxicity

Although the use of the VAS is a validated method of determining tolerability, this method can become subjective. Therefore, a reviewed method of the VAS tool should be considered in future work.

5.4. Future work

One of the covariates in determining the tolerability of PAS was the use of other concomitant medications. It would therefore we worthwhile investigating a control group receiving second-line agents without PAS, to clarify the extent of intolerance attributed to PAS administration, and to eliminate other second-line agents such as capreomycin and ethionamide, which are known to have similar toxic effects.

References

1. Lehmann J. The role of the metabolism of p-aminosalicylic acid (PAS) in the treatment of tuberculosis. Interaction with the metabolism of isonicotinic acid hydrazide (INH) and the synthesis of cholesterol. Scand J Respir Dis. 1969; 50(3):169-185.
2. Lehmann J. The Treatment of Tuberculosis in Sweden with Para-Aminosalicylic Acid (PAS): A Review. Dis Chest. 1949; 16(6):684–703.
3. Medical Research Council. The prevention of streptomycin resistance by combined chemotherapy. Subcommittee of the Medical Research Council's Streptomycin Tuberculosis Association. Br Med J. 1952; 2:735-746.
4. Todd RM. Treatment of primary pulmonary tuberculosis with PAS. Br Med J. 1953; 1:1247–1249.
5. Jindani A, Aber VR, Edwards EA, Mitchison DA. The early bactericidal activity of drugs in patients with pulmonary tuberculosis. Am Rev Respir Dis. 1980; 121(6):939–949.
6. Donald PR, Diacon AH. The early bactericidal activity of anti-tuberculosis drugs: a literature review. Tuberculosis. 2008; 88:S75--S83.
7. Momekov G, Momekova D, Stavrakov G. Para-Aminosalicylic Acid–Biopharmaceutical, Pharmaco-Logical, and Clinical Features and Resurgence As an Anti-tuberculosis agent. Pharmacia. 2015; 62(1):25–51.
8. Fox W, Ellard GA, Mitchison DA. Studies on the treatment of tuberculosis undertaken by the British Medical Research Council Tuberculosis Units, 1946-1986, with relevant subsequent publications. Vol. 3, International Journal of Tuberculosis and Lung Disease. 1999.
9. Riska N. Variations in the PAS concentration in the blood and their influence on treatment of tuberculosis. Acta Taabercuulosea Scand. 1954; 30:144–163.
10. Riska N, Tennberg C. Optimal PAS dosage. Am Rev Respir Dis. 1962; 86(3):430–433.
11. Riska N. PAS therapy with a daily unfractionated dose. Acta Tuberc Scand. 1959; 37:104–111.
12. Yue WY, Cohen SS. Toleration and Absorption of Sodium Para-Aminosalicylate and Para-Aminosalicylic Acid (Neopasalate). Dis Chest. 1966; 49(2):165–174.

13. Shean K, Streicher E, Pieterson E, Symons G, van Zyl Smit R, Theron G, et al. Drug-Associated Adverse Events and Their Relationship with Outcomes in Patients Receiving Treatment for Extensively Drug-Resistant Tuberculosis in South Africa. PLoS One. 2013;8(5):

14. Sy SKB, De Kock L, Diacon AH, Werely CJ, Xia H, Rosenkranz B, et al. N-acetyltransferase genotypes and the pharmacokinetics and tolerability of para-aminosalicylic acid in patients with drug-resistant pulmonary tuberculosis. Antimicrob Agents Chemother. 2015; 59(7):4129–4138.

15. Peloquin CA, Henshaw TL, Huitt GA, Berning SE, Nitta AT, James GT. Pharmacokinetic evaluation of para-aminosalicylic acid granules. Pharmacother J Hum Pharmacol Drug Ther. 1994; 14(1):40–46.

16. Peloquin CA. Therapeutic drug monitoring in the treatment of tuberculosis. Drugs. 2002;62(15):2169–2183.

17. De Kock L, Sy SKB, Rosenkranz B, Diacon AH, Prescott K, Hernandez KR, et al. Pharmacokinetics of para-aminosalicylic acid in HIV-uninfected and HIV-coinfected tuberculosis patients receiving antiretroviral therapy, managed for multidrug-resistant and extensively drugresistant tuberculosis. Antimicrob Agents Chemother. 2014; 58(10):6242–6250.

18. Bang HO, Jacobsen LK, Strandgaard E, Yde H, others. Metabolism of isoniazid and para-amino salicylic acid (PAS) in the organism and its therapeutic significance. Acta Tuberc Pneumol Scand. 1962; 41(4):237–251.

19. Donald PR, Diacon AH. Para-aminosalicylic acid: The return of an old friend. Lancet Infect Dis. 2015; 15(9):1091–1099.

20. Cummins CL, O'Neil WM, Soo EC, Lloyd DK, Wainer IW. Determination of p-aminosalicylic acid and its N-acetylated metabolite in human urine by capillary zone electrophoresis as a measure of in vivo N-acetyltransferase 1 activity. J Chromatogr B Biomed Appl. 1997; 697(1–2):283–288.

21. Peloquin CA, Berning SE, Huitt GA, Childs JM, Singleton MD, James GT. Once-daily and twice-daily dosing of p-aminosalicylic acid granules. Am J Respir Crit Care Med. 1999; 159(3):932–934.

22. Peloquin CA, Zhu M, Adam RD, Singleton MD, Nix DE. Pharmacokinetics of para-aminosalicylic acid granules under four dosing conditions. Ann Pharmacother. 2001;

35(11):1332–1338.

Appendices

Appendix 1: Individual pharmacokinetic parameters of study subjects.

Individual pharmacokinetic parameters for PAS in patients on the 8g once-daily GSR-PAS and 4g twice-daily GSR-PAS dosing regimens.

Subject	PK parameter					
	8g once-daily			4g twice daily		
	C_{max} (mg/L)	AUC_{0-24} (hr*mg/L)	T_{max} (hr)	C_{max} (mg/L)	AUC_{0-24} (hr*mg/L)	T_{max} (hr)
1	70.64	721.32	4	29.62	265.79	4
2	52.52	610.84	12	45.48	370.72	3
3	98.74	1185.98	8	53.63	817.56	6
4	46.34	450.02	4	39.42	322.41	4
5	69.08	724.23	3	40.42	703.42	12
6	102.41	1295.97	8	54.57	663.94	3
7	38.29	487.92	24	72.39	1219.75	8
8	91.58	950.93	3	49.31	311.76	2
9	62.43	813.84	8	76.31	766.35	8
10	85.1	1274.97	12	51.13	656.19	2
11	66.69	827.75	8	31.77	363.42	3
12	131.49	1580.68	8	89.17	1245.94	2
13	89.9	1105.4	4	68.55	487.7	3
14	107.94	1144.05	8	53.82	825.23	8
15	82.69	987.96	4	41.48	609.55	4
17	66.11	804.99	8	26.15	432.61	8
18	150.94	1922.41	8	92.06	1337.02	8
19	72.89	479.85	8	21.76	260.83	12
20	135.99	1702.59	6	113.01	1459.56	6
21	124.6	1435.82	8	63.7	578.58	6
22	85.61	851.19	6	65.04	934.95	6
23	94	1271.98	8	56.9	614.55	6
24	46.04	247.94	8	missing	missing	missing
25	77.05	1098.23	8	missing	missing	missing
26	missing	missing	Missing	72.02	595.29	2
27	52.25	424.15	4	missing	missing	missing
28	51.64	587.26	12	missing	missing	missing
29	missing	missing	Missing	50.85	527.41	3
30	missing	missing	missing	35.37	283.19	6
31	142.62	1962.72	6	missing	missing	missing

Individual pharmacokinetic parameters for APAS in patients on the 8g once-daily GSR-PAS and 4g twice-daily GSR-PAS dosing regimens.

Subject	PK parameter					
	8g once-daily			4g twice daily		
	C_{max} (mg/L)	AUC_{0-24} (hr*mg/L)	T_{max} (hr)	C_{max} (mg/L)	AUC_{0-24} (hr*mg/L)	T_{max} (hr)
1	25.87	443.59	6	22.97	341	6
2	17.46	227.53	6	14.89	196.96	4
3	11.92	197.05	12	14.59	268.44	4
4	9.78	163.68	8	7.59	135.03	3
5	11.31	199.33	12	14.61	262.78	6
6	7.97	128.06	8	8.96	176.65	2
7	19.75	387.05	4	20.67	407.65	6
8	24.37	441.46	6	34.54	294.92	8
9	20.3	320.5	6	14.02	268.19	3
10	40.93	845.02	8	28.05	540.43	8
11	10.69	173.2	6	11.8	165.83	6
12	14	239.08	4	9.81	186.68	4
13	11.57	217.28	8	11.29	186.85	4
14	27.01	393.76	8	25.38	418.91	8
15	13.38	271.09	12	12.89	235.28	8
17	14.19	216.98	8	15.03	234.08	4
18	18.43	291.64	8	22.51	383.76	6
19	9.04	122.59	8	10.14	178.57	6
20	31.83	507.2	6	29.6	517.59	6
21	16.36	212.25	6	7.94	157.97	8
22	19.87	255.91	8	15.57	250.33	6
23	20.96	317.76	6	22.2	146.76	8
24	14.1	77.36	8	missing	missing	missing
25	10.93	185.36	6	missing	missing	missing
26	missing	missing	missing	8.95	146.76	2
27	10.72	156.88	12	missing	missing	missing
28	16.74	248.87	8	missing	missing	missing
29	missing	missing	missing	12.15	312.99	8
30	missing	missing	missing	4.99	214.41	6
31	19.23	376.07	24	missing	missing	missing

Individual pharmacokinetic parameters for GPAS in patients on the 8g once-daily GSR-PAS and 4g twice-daily GSR-PAS dosing regimens.

Subject	PK parameter					
	8g once-daily			4g twice daily		
	C_{max} (mg/L)	AUC_{0-24} (hr*mg/L)	T_{max} (hr)	C_{max} (mg/L)	AUC_{0-24} (hr*mg/L)	T_{max} (hr)
1	9.1	75.41	4	3.82	32.28	4
2	2.77	33.14	12	2.07	23.65	3
3	6.4	72.94	3	3.89	64.95	24
4	7.62	74.23	6	6.65	52.05	4
5	3.8	45.65	3	3.01	50.6	24
6	6.08	77.1	8	5.06	60.93	3
7	10.7	106.47	24	14.06	269.31	8
8	29.11	248.58	3	9.95	56.64	2
9	9.45	89.91	4	4.47	63.45	6
10	17.16	261.33	12	17.39	171.56	24
11	4.56	52.95	8	5.02	65.65	3
12	5.73	89.69	8	6.79	69.02	2
13	20.51	236.51	6	16.8	143.12	4
14	6.14	74.66	3	4.87	71.29	24
15	11.98	151.46	4	7.31	99.62	4
17	3.29	39.73	8	2.43	34.97	2
18	5.76	78.6	8	5.72	86.15	8
19	3.01	24.96	8	1.08	19.47	12
20	8.65	118.1	6	8.54	130.9	6
21	5.89	67.88	8	8.07	80.46	4
22	5.29	41.38	3	4.39	60.01	3
23	3.6	54.1	12	2.93	29.96	3
24	5.18	11.62	8	missing	missing	missing
25	6.84	99.7	8	missing	missing	missing
26	missing	missing	missing	7.96	76.49	3
27	5.17	31.83	4	missing	missing	.missing
28	2.34	27.14	12	missing	missing	missing
29	missing	missing	missing	4.37	41.93	3
30	missing	missing	missing	4.99	47.96	6
31	14.55	228.05	8	missing	missing	missing

Approval Notice
New Application

25-July-2017

Ethics Reference #: S17/05/107

Title: PHARMACOKINETIC STUDY OF ACETYL-PAS AND GLYCINE-PAS AND THE CORRELATION BETWEEN THE RATE OF METABOLISM AND THE DEVELOPMENT OF TOXICITY IN MDR- and XDR-TB PATIENTS TREATED WITH PAS

Dear Ms K Adams

The **New Application** received on **17-May-2017** was reviewed by members of **Health Research Ethics Committee (HREC) 1** via **expedited** review procedures on **24-July-2017** and was approved.

Please note the following information about your approved research protocol:

Protocol Approval Period: **24-July-2017 – 23-July-2018**

Please remember tou se your protocol number (S17/05/107) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired.
The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.
Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No. 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki and the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles, Structures and Processes 2015 (Departement of Health).

Addendums

Addendum A

Quantification of esomeprazole and its three major metabolites in human plasma by UHPLC-MS/MS using a simple sample extraction method

6.1 Introduction

Esomeprazole is chemically (*S*)-5-methoxy-2-[(4-methoxy-3-5-dimethylpyridin-2-yl) methylsulfinyl)]-3-H-benzoimidazole (**Figure 4.1**), and one of the optical isomers of its counterpart omeprazole.[1–3] Esomeprazole is part of a class of drugs classified as proton pump inhibitors (PPI's), which reduce gastric acid secretion by inhibiting the hydrogen/potassium adenosinetriphosphate enzyme (H^+, K^+ -ATPase).[4,5] and has shown to be clinically effective and safe in the treatment of gastric acid-related diseases such as gastroesophageal reflux disease (GERD), non-steroidal anti-inflammatory drugs (NSAID's) induced gastric intestinal symptoms, *Helicobacter pylori* infection, peptic ulcers and Zollinger-Ellison syndrome.[1,6–8]

Similar to other PPI's, esomeprazole is a prodrug that is converted to its active inhibitor sulphonamide, in the acidic compartment of the parietal cells.[9] For this reason it is formulated as a delayed-release capsule to prevent the rapid dissolution in the acidic gastric cavity, during absorption.[6,10] The pharmacokinetic profile of esomeprazole is widely described.[3,9,11,12] It is highly bound to plasma proteins (97%) and metabolised by two cytochrome P450 (CYP) isoenzymes namely, CYP3A4 and CYP2C19, through sulfoxidation and hydroxylation, respectively. The major metabolites present in plasma are 5-hydroxy esomeprazole, omeprazole sulfone and a minor metabolite 5-*O*-desmethyl esomeprazole (**Figure 6.1**). According to clinical studies, the overall metabolism relies more on the activity of CYP2C19 than that of CYP3A4.[2,13]

The rate of metabolism of esomeprazole is known to be lower and less variable than its optical enantiomer, (*R*)-omeprazole and its racemate.[12] This is supported by *in vivo* studies

of healthy individuals, where the plasma concentrations of esomeprazole were higher than those of omeprazole.[4] Furthermore, a higher area under the concentration time curve (AUC) of almost two fold than that of omeprazole was observed in patients with GERD.[8] As a result of this pharmacokinetic profile for esomeprazole, it is considered to be a better choice in the treatment of acid related diseases.[3,5]

A number of methods have been developed to assay esomeprazole in biological samples, using liquid chromatography tandem mass spectrometry (LC-MS/MS).[1,14–16] Methods proposed by Hultman *et al.*[14] for quantification of esomeprazole including two metabolites (5-hydroxyesomeprazole, omeprazole sulfone) in human, rat and dog plasma and Mogili *et al.*[15] for determination of esomeprazole in human plasma required lengthy chromatography run times, and lacked sensitivity. Gopanith *et al.*[16] developed a more sensitive method for determination of esomeprazole in human plasma applying an expensive sample preparation technique using solid phase extraction (SPE). The method published by Chunduri *et al.*[1] quantified esomeprazole and another PPI using the superior ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) system, which offered several advantages over previously discussed techniques including the use of a liquid-liquid extraction (LLE) sample preparation method.

To the best of our knowledge, no UPLC-MS/MS method has been developed to assay esomeprazole and its three metabolites simultaneously in human plasma. The current study describes a rapid and sensitive method, which employed a protein precipitation (PPT) technique for sample extraction. Ultra performance liquid chromatography with electrospray ionization (ESI) and tandem mass spectrometry is applied to quantify esomeprazole and its main metabolites, 5-hydroxyesomeprazole, omeprazole sulfone and 5-*O*-desmethyl

omeprazole simultaneously in a short run time (4 minutes), wide linearity range, and a simple reproducible extraction method that is cost effective.

The aim of the study was to develop and validate the presented method according to bio-analytical guidelines set out by the Food and Drug Administration.[17] Application of the assay included a clinical pharmacokinetic study following oral administration of 40mg of esomeprazole to pregnant women diagnosed with pre-eclampsia as described by Cluver *et al.*[18] in the American Journal of Obstetrics and Gynecology. The latter study was conducted to determine whether esomeprazole prolonged gestation in pregnancies with pre-eclampsia and if it could decrease circulating soluble fms-like tyrosine kinase 1 (sFlt-1)[19] which is released by placental tissue and considered to be responsible for causing widespread maternal endothelial dysfunction and organ injury associated with pre-eclampsia.[19]

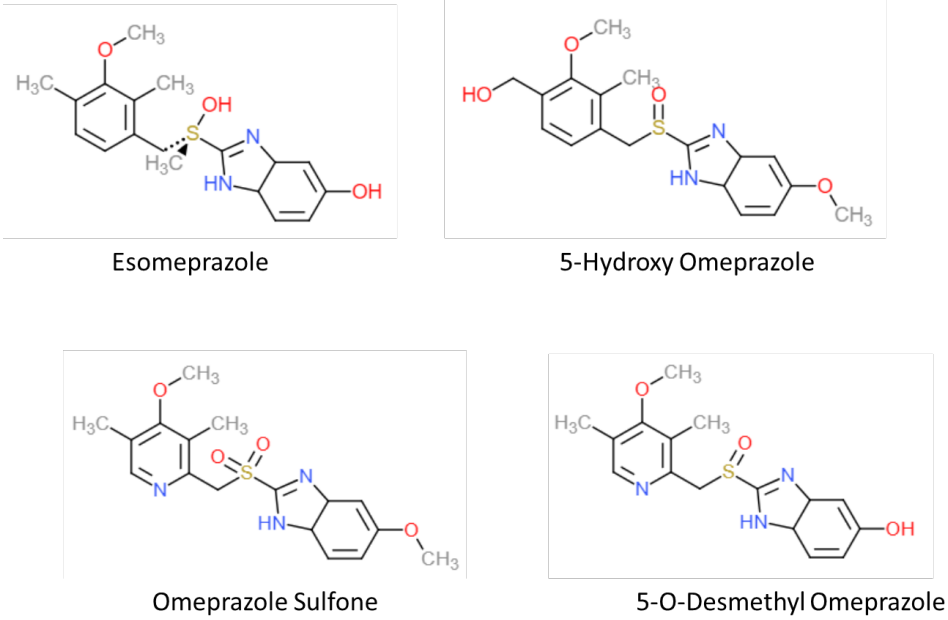

Figure 6.1: Chemical structures of (*S*)-enantiomer esomeprazole and the three metabolites 5-hydroxy omeprazole, omeprazole sulfone, and 5-*O*-desmethyl omeprazole. Adapted from Abelo *et al.*[12]

6.2 Experimental methods

6.2.1 Chemicals and Reagents

All reference standards including esomeprazole magnesium hydrate, 5-hydroxy omeprazole, omeprazole sulfone, 5-*O*-desmethyl omeprazole, and a deuterated internal standard d3-esomeprazole were purchased from Sigma Aldrich (St Louis, MO, USA). Analytical grade acetonitrile and methanol were from ROMIL Ltd (Cambridge, England). Water was purified through a Milli-Q water purification system (Milford, MA, USA) and used throughout the study. Drug-free human plasma was obtained from Tygerberg Hospital Blood Bank (Western Cape, South Africa), and heparinized prior to use.

6.2.2 Preparation of calibration standards and quality control samples

Stock solutions of esomeprazole, 5-hydroxy omeprazole, omeprazole sulfone, 5-*O*-desmethyl omeprazole, and d3-esomeprazole were prepared separately by dissolving requisite amounts in acetonitrile:water (70:30 v/v) in volumetric flasks to achieve a primary concentration of 200 µg/mL respectively. These analytes were then combined, excluding the internal standard, by serial dilution with blank plasma to generate calibration standards in the concentration range of 0.001 – 2.5 µg/mL. Quality control (QC) samples were prepared similarly at concentrations of 3.7 µg/mL (high quality control, HQC), 0.37 µg/mL (medium quality control, MQC), 0.106 µg/mL (low quality control, LOQ), and 0.014 µg/mL (lower limit of quality control, LLOQ).

Working solution (0.2 µg/mL) of the internal standard was prepared by diluting the stock solution (200 µg/mL) in acetonitrile.

6.2.3 Sample preparation

A simple protein precipitation method was developed for the extraction of the analytes and internal standard from human plasma. Prior to analysis, all frozen calibration standards and subject samples were thawed at room temperature. Aliquots of 100 µL of each subject samples, standards, QC's and blank plasma were dispensed into 2 mL microcentrifuge tubes. Then, a volume of 900 µL extraction solvent containing internal standard (0.1 µg/mL) in acetonitrile:2 mM ammonium formate buffer (50:50 v/v) at pH 5.5, was added to the sample tube for protein precipitation. The mixture was vortexed for 10 min, and centrifuged at 11000 rotations per minute (rpm) for 3 minutes. Eight hundred micro litres (800 µL) of supernatant was transferred to a suitable auto sampler vial and sealed cap, before storage in a refrigerator at 4 ºC prior to analysis within 1 day.

6.2.4 LC-MS/MS instrumentation and conditions

Liquid chromatography was performed using a Waters Xevo TQS triple quadrupole mass spectrometer connected to a Waters Acquity Ultra performance liquid chromatograph (UPLC) system (Waters Corporation, Milford, MA, USA) equipped with a binary solvent pump and temperature controlled sample organizer. Quantification of the analytes and IS were achieved by operating the mass spectrometer in positive ion electrospray ionization (ESI) at a desolvation temperate of 400 ºC, desolvation gas as nitrogen at a flow rate of 800 L/h and capillary voltage of 3.5 kV. Multiple reaction monitoring mode (MRM), summarized in **Table 6.1**, was used for data acquisition. Separation was achieved using a Waters HSS T3 column (1.7 µm particle size, 2.1 x 50 mm), maintained at column temperature of 40°C and

flow rate of 0.5 mL/min with a binary gradient method. Mobile phase A contained 0.1% formic acid in water while mobile phase B had 0.1% formic acid in acetonitrile. The gradient started with 0.3 minutes of 95% mobile phase A followed by a linear gradient over 1.7 minutes of 62% mobile phase A and a wash step of 0.5 minutes with 100% mobile phase B.

Table 6.1: Multiple reaction monitoring (MRM) conditions of each analyte and internal standard.

Analyte	M+H	Retention Time (minutes)	Cone voltage (V)	Quantifier product ion (Collision energy)	Qualifier product ion (Collision Energy)
Esomeprazole	346.2	1.9	20	136.1 (25)	151.2 (20)
5-Hydroxy Omeprazole	362	1.7	15	152 (25)	167 (20)
Omeprazole Sulfone	362	2.2	15	150 (25)	168 (20)
5-*O*-Desmethyl Omeprazole	332	1.5	10	151 (15)	198 (10)
d3-Esomeprazole	349	1.9	15	151 (20)	198 (15)

6.2.5 Method validation

All validation procedures were performed according to U.S. Food and Drug Administration (FDA)[17] industry guidance for the validation of bio-analytical methods.

6.2.5.1 Selectivity and Specificity

Selectivity of the method is accepted as lack of interference by endogenous plasma components and/or absence of co-eluting peaks in the same MRM channel. This was

evaluated by comparing the chromatograms of numerous placebo samples included in the trial, which were spiked with all analytes.

6.2.5.2 Accuracy and Precision

The method was evaluated for accuracy and precision by 6x injection of quality control (QC) samples at four different concentrations (LLOQ, LOQ, MQC, HQC) within a batch for intra-day accuracy. A QC and blank sample was included after every 20 injections. Inter-day batch accuracy and precision was determined at the same QC concentrations on three different days. Acceptance criteria included percentage recovery as the difference between the mean measured values and experimental value within ± 15% deviation at higher concentrations and ± 20% at the LLOQ. Precision was recorded as a percentage of relative standard deviation (%RSD), with the same criteria.

6.2.5.3 Linearity and LLOQ

Calibration standards were prepared by dilution of analyte stock solutions with blank plasma proceeded with IS, in a concentrations range of 0.001 – 2.5 µg/mL which included the LLOQ. The curves were constructed by plotting the peak area ratio (y-axis) of each analyte to the internal standard, versus, the corresponding concentrations (x-axis). Acceptance criteria for each back calculated standard concentration was ± 15% deviation from the nominal value, and ± 20% at the LLOQ value.

The lowest standard on the curve where the analyte peak should be identifiable, discrete and reproducible was considered as the LLOQ. Accepted criterion included precision of 20% and an accuracy of 80-120% at this value.

6.3 Results and Discussion

In an effort to simultaneously determine the pharmacokinetics of esomeprazole and its metabolites, we developed a sensitive and high throughput assay using UPLC-MS/MS. To address this objective, parameters attaining to chromatography, mass spectrometry and sample extraction conditions were evaluated and are reported below.

6.3.1 Optimization of chromatographic conditions

Mass spectrometry conditions were optimization to achieve the maximum stable signal of the precursor ions and corresponding product ions of the analytes in question. Using an electrospray ionization source, tuning of the MS parameters was performed in positive and negative ionization for each analyte and IS. Better intensity was observed in the positive mode, with Q1 MS scan dominated by protonated precursor ions *m/z* 364.2, 362, 362, 332 and 349 for esomeprazole, 5-hydroxy omeprazole, omeprazole sulfone, 5-*O*-desmethyl omeprazole and IS, d3-esomeprazole respectively. These precursor ions were used to obtain the Q3 product ion spectra, summarized in **Table 6.1**. Hereby, the mass transition ion pair was selected as *m/z* 346.2>136.1; 346.2>151.2 for esomeprazole, *m/z* 362>152; 362>167 for 5-hydroxy omeprazole, *m/z* 362>150; 362>168 for omeprazole sulfone, *m/z* 332>151; 332>198 for 5-*O*-desmethyl omeprazole, and *m/z* 349>151; 349>198 for IS, d3-esomeprazole (**Figure 6.2**). The characteristic selectivity of using MS/MS detection was expected to be

advantageous in developing a selective and sensitive method. Collision energy and cone voltage were optimized to get the highest intensity signal for these precursor and product ions.

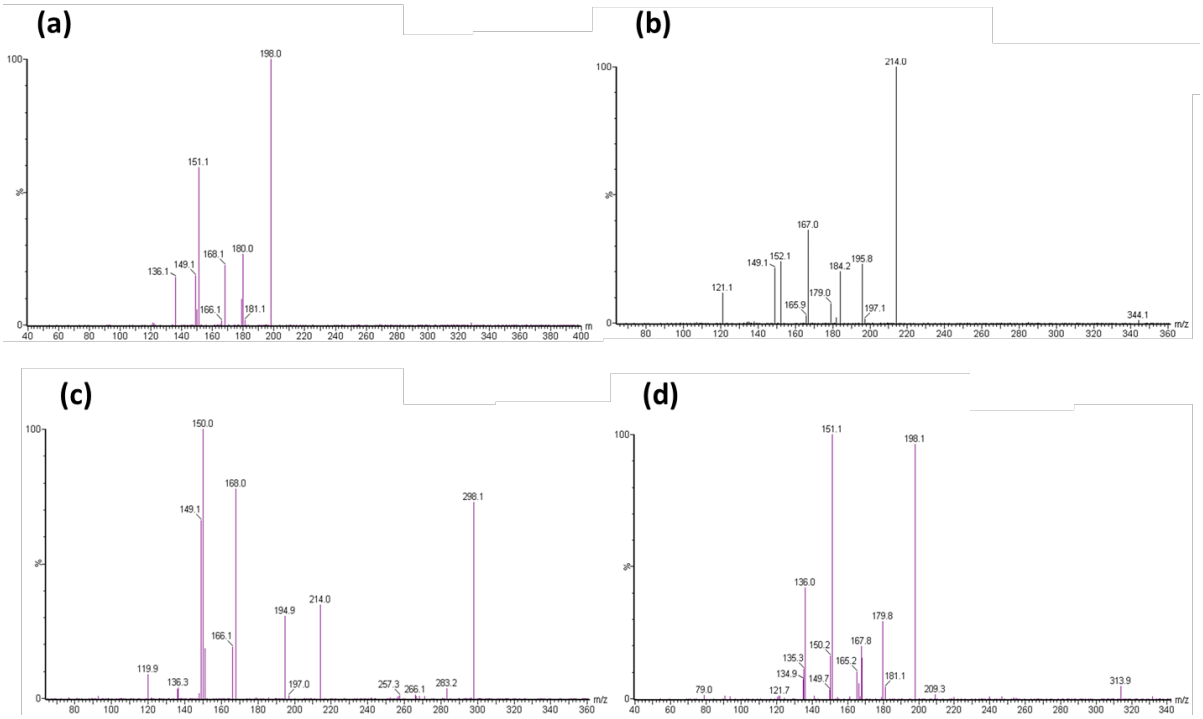

Figure 6.2: Product ion mass spectra of (a) esomeprazole, (b) 5-hydroxy omeprazole, (c) omeprazole sulfone, and (d) 5-*O*-desmethyl omeprazole.

Composition and nature of the mobile phase together with different columns were optimized in order to achieve good chromatographic resolution and symmetrical peak shapes within a short run time. The viability of several solvents including acetonitrile and methanol with different buffers including ammonium acetate, and ammonium formate were evaluated on C_{18} columns through many experiments, to determine the best resolution of all analytes and IS from any interfering biological components. Good separation and elution were achieved with a gradient mobile phase system consisting of 0.1% formic acid solution (A) to acetonitrile containing 0.1% formic acid (B) solution, for a total run time of 3.5 minutes carried out on a Waters HSS T3 (1.7 µm, 2.1 x 50 mm) column. The chromatography time included diversion

of flow from the LC to waste for the first minute of the run. The retention times are summarized in **Table 6.1**, for each analyte including the IS.

6.3.2 Selectivity

The placebo samples were spiked with LLOQ working solutions, which included internal standard in order to confirm the lack of interference at the analyte's specific retention times and the absence of lot-to-lot variation, as shown by **Figure 6.3**.

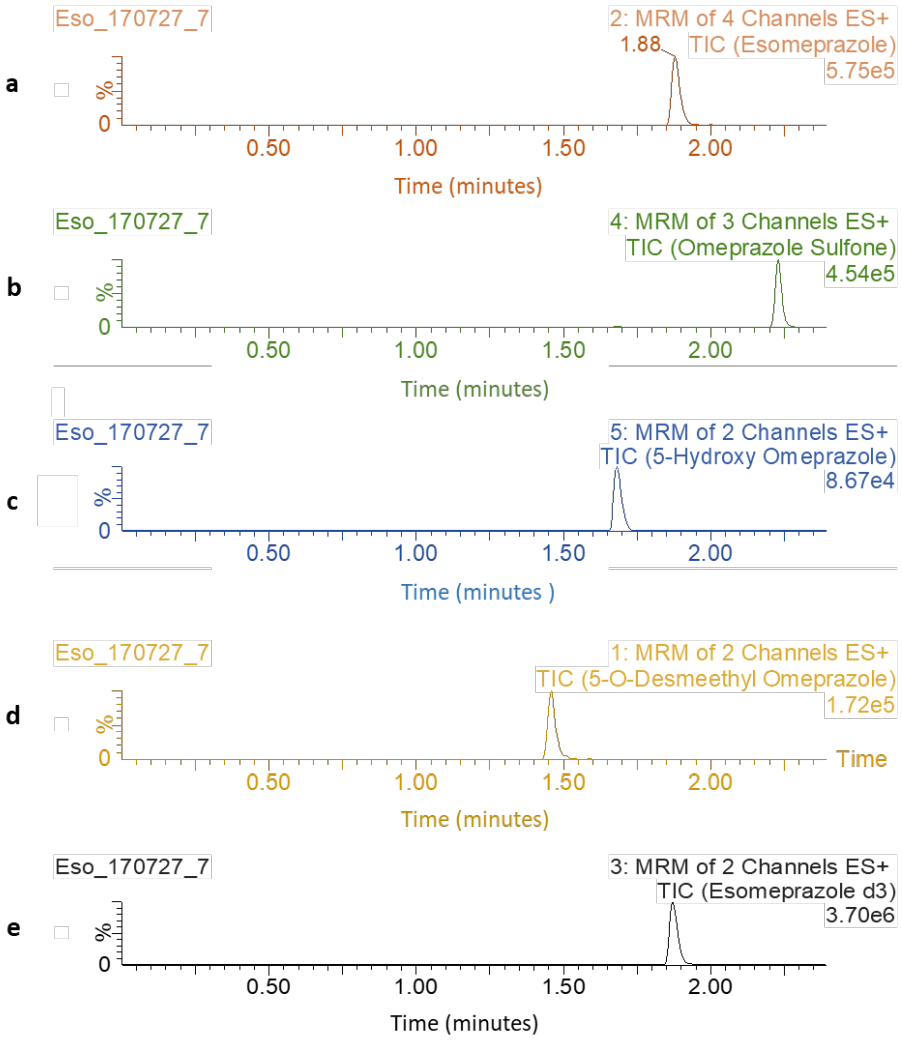

Figure 6.3: Typical chromatogram of analytes (a) esomeprazole, (b) omeprazole sulfone, (c) 5-hydroxy omeprazole, (d) 5-*O*-desmethyl omeprazole, and (e) IS, d3-esomeprazole in human blank plasma spiked with LLOQ standard.

6.3.3 Accuracy and Precision

The intra- and inter-day precision and accuracy values were within the acceptance limit for all the analytes and are summarized in **Table 6.2**. The intra-day accuracy ranged between 83% and 104.5% with a precision of 1.7% - 10.5%, while the inter-day accuracy was between 80.0% and 113% with a precision of 5.3% - 20%.

Adequate sensitivity was observed for the LLOQ control samples for all analytes. The intra-day precision ranged from 2.6% to 10.5%, while the accuracy was between 80% and 111%.

6.3.4 Linearity and LLOQ

The nine-point calibration curve was found to be linear over the concentration range of 0.001 – 2.5 µg/mL for all four analytes, as summarized in **Table 6.3**. The accuracy values ranged from 97.5% to 104.5% for esomeprazole, 98.3% to 110% for 5-hydroxy omeprazole, 77.9% to 110% for omeprazole sulfone, and 73.6% to 108% for 5-*O*-desmethyl omeprazole. Precision (%RSD) values vary from 1.3% to 12% for esomeprazole, while more variation was observed for the metabolites ranging from 1.3% to 20%.

Table 6.2: Intra- and inter-batch precision and accuracy of all analytes (mean µg/mL, RSD %, accuracy %).

Analyte	Theoretical Concentration (µg/mL)		Intra-day (n=6)			Inter-day (n=6)		
			Mean (µg/mL)	RSD (%)	Accuracy (%)	Mean (µg/mL)	RSD (%)	Accuracy (%)
Esomeprazole								
	LLOQ	0.014	0.014	2.6	103.4	0.011	11.8	94
	LQC	0.106	0.107	2.4	97.5	0.120	12.9	112
	MQC	0.37	0.37	1.7	99.3	0.371	10.4	100.2
	HQC	3.7	3.7	2.0	100.6	3.7	5.5	98.8
5-Hydroxy Omeprazole								
	LLOQ	0.014	0.014	5.6	99.8	0.012	15	85
	LQC	0.106	0.109	9.1	99	0.121	11.8	113
	MQC	0.37	0.38	2.3	104.5	0.35	9.9	95.8
	HQC	3.7	3.5	9.4	93.6	3.5	8.7	94.3
Omeprazole Sulfone								
	LLOQ	0.014	0.014	2.6	103.4	0.011	11	80
	LQC	0.106	0.107	2.4	97.5	0.103	10.4	96
	MQC	0.37	0.37	1.7	99.3	0.38	10.2	104
	HQC	3.7	3.3	3.4	96.3	3.8	6.2	102.6
5-*O*-Desmethyl Omeprazole								
	LLOQ	0.014	0.011	10.5	83	0.015	20	111
	LQC	0.106	0.109	5.5	99.2	0.120	14	112
	MQC	0.37	0.38	2.4	102.1	0.36	10	100.3
	HQC	3.7	3.8	1.7	101.8	3.7	5.3	99.7

LLOQ = lower limit of quantification; **LQC** = low quality control; **MQC** = medium quality control; **HQC** = high quality control.

Table 6.3: Linearity of calibration standards (mean µg/mL, RSD %, accuracy %).

(µg/mL)	Esomeprazole (n=6)			5-Hydroxy Omeprazole (n=6)			Omeprazole Sulfone (n=6)			5-O-Desmethyl Omeprazole (n=6)		
	Mean (µg/mL)	RSD (%)	Accuracy (%)	Mean (µg/mL)	RSD (%)	Accuracy (%)	Mean (µg/mL)	RSD (%)	Accuracy (%)	Mean (µg/mL)	RSD (%)	Accuracy (%)
0.001	0.002	12.0	102.5	0.002	9.0	106.0	0.001	12.0	77.9	0.001	20	73.6
0.027	0.027	1.8	99.2	0.028	8.5	101.6	0.027	1.8	99.2	0.028	8	103.5
0.055	0.054	1.8	98.0	0.054	9.0	98.3	0.054	1.8	98.0	0.055	4.6	100.7
0.165	0.134	1.3	98.7	0.169	6.3	102.7	0.162	1.3	98.7	0.164	8.5	99.5
0.246	0.243	2.1	98.9	0.259	6.4	105.4	0.243	2.1	98.9	0.253	4.5	103.1
0.555	0.565	1.6	101.8	0.557	5.8	105	0.565	1.6	101.8	0.568	4.4	102.4
0.833	0.852	1.9	102.3	0.920	2.3	110	0.852	2.5	102.3	0.892	3.9	107
1.25	1.31	2.5	104.5	1.29	9.4	103.8	1.31	2.5	105	1.35	5.3	108
2.5	2.4	3.1	97.5	2.7	7.7	106	2.4	3.1	97.5	2.4	3.4	96

6.3.5 Sample extraction

A crucial part of the study was to develop a simple, reliable and cost effective extraction method to analyse esomeprazole and its metabolites in human plasma. Therefore, the extraction procedure was investigated as the first priority. Protein precipitation (PPT) experiments using ammonium formate-acetonitrile (50:50 v/v) and ammonium formate-acetonitrile (70:30 v/v) were compared over a period of 3 days. A concentration of 2 mM ammonium formate showed to stabilize the compounds better than the previously reported use of 5 mM ammonium formate.[15] The area response for esomeprazole was similar

between the two ratios, but the 70:30 ratio was not optimal for the metabolites. For this reason the final optimized extraction solvent was 2 mM ammonium formate-acetonitrile (50:50 v/v). It was noted that once extracted, the samples needed to be cooled down before centrifugation and LCMS analysis. PPE showed to be suitable in recovery percentage and reproducibility. This type of extraction protocol is useful in saving costs on solvents and extraction time necessary for clinical sample analysis.

6.3.6 Stability

The stability of esomeprazole is pH dependent, as it rapidly degrades in acidic environments. It has acceptable stability under alkaline conditions. At pH of 6.8, the half-life is approximately 19 hours and 8 hours at 25ºC and 37 ºC respectively.[20]

6.3.7 Robustness

To evaluate robustness of the method, analysis was performed by two separate analysts, sites, machines and chromatographic conditions. The results were comparable within the acceptance range of 15% in accuracy and precision.

6.3.8 Application of method in pharmacokinetic study

The presented UPLC-MS/MS method was successfully used to quantify esomeprazole and its three metabolites in human plasma collected from pregnant volunteers administered esomeprazole (n=10) or placebo (n=9) and umbilical cord blood collected at birth (n=82). Pharmacokinetic analysis was conducted on maternal samples only, collected at 15, 30, and

45 minutes (pre-dose) and at 1, 1.5, 2, 4, 8, 24 hours (post-dose). With such a large number of samples, it was necessary for the assay to be rapid. All clinical samples were quantifiable using the described precipitation extraction protocol and LC-ESI-MS/MS system. The area under plasma concentration time curves (AUC_{0-24}) investigated for esomeprazole and each of its metabolites are presented in Cluver *et al*.[18] Esomeprazole exposure levels were in the same range as previously reported.[1] Metabolites 5-hydroxy and 5-*O*-desmethyl were present in low concentrations, and much lower than the parent drug. Overall, omeprazole sulfone displayed higher levels among the metabolites.

6.4 Summary and Conclusion

The method development is reliable for determination of esomeprazole and metabolites 5-hydroxy, sulfone, and 5-*O*-desmethyl, within a single run. This method can be applied for the rapid clinical monitoring of esomeprazole in human plasma, under 4 minutes per run. It makes use of a simple and efficient sample clean up using protein precipitation and sample volume of 100 µL plasma. Method validation performed on this method supports its high precision and accuracy, at the LLOQ of 0.001 µg/mL, and a wide linear range. All performance characteristics evaluated proved to be within the accepted criteria as per the FDA bioanalytical guidelines. Less precision and accuracy was observed for the metabolites when compared with esomeprazole, as a result of the low concentrations these metabolites are present. Additionally, this method showed to be sufficient for use in bioequivalence and other pharmacokinetic studies following oral administration of esomeprazole in human subjects.

References

1. Chunduri RHB, Dannana GS. Development and validation of a high throughput UPLC–MS/MS method for simultaneous quantification of esomeprazole, rabeprazole and levosulpiride in human plasma. J Pharm Anal. 2016; 6(3):190–198.
2. Andersson T. Pharmacokinetics, metabolism and interactions of acid pump inhibitors. Clin Pharmacokinet. 1996; 31(1):9–28.
3. Hassan-Alin M, Andersson T, Bredberg E, Rohss K. Pharmacokinetics of esomeprazole after oral and intravenous administration of single and repeated doses to healthy subjects. Eur J Clin Pharmacol. 2000; 56(9–10):665–670.
4. Andersson TB, Antonsson M, Naudot AK, Abel A, Astrazeneca R, Mo D. Stereoselective Metabolism of Omeprazole By Human Cytochrome P450 Enzymes Abstract : 2000; 28(8):966–972.
5. Scott LJ, Dunn CJ, Mallarkey G, Sharpe M. Esomeprazole. Drugs. 2002; 62(10):1503–1538.
6. Sathiyaraj M, Vijay AR, N S. Bioanalytical Method Development and Validation of Esomepraole in Human Plasma by LCMS/MS. Asian J Res Chem. 2010; 3(2):477–484.
7. Vakil NB, Shaker R, Johnson DA, Kovacs T, Baerg RD, Hwang C, et al. The new proton pump inhibitor esomeprazole is effective as a maintenance therapy in GERD patients with healed erosive oesophagitis: a 6-month, randomized, double-blind, placebo-controlled study of efficacy and safety. Aliment Pharmacol Ther. 2001; 15(7):927–935.
8. Lind T, Rydberg L, Kylebäck A, Jonsson A, Andersson T, Hasselgren G, et al. Esomeprazole provides improved acid control vs. omeprazole In patients with symptoms of gastro-oesophageal reflux disease. Aliment Pharmacol Ther. 2000; 14(7):861–867.
9. Andersson T, Hassan-alin M, Hasselgren G, Röhss K, Weidolf L, Pharmacology C, et al. Pharmacokinetic Studies with Esomeprazole , the (S) -Isomer of Omeprazole. Clin Pharmacokinet. 2001; 40(6):411–426.
10. Ullah MA, Shams-Ud-Dowla, Maruf A Al, Azad MAK, Shohag MH, Sultana R, et al.

Relative bioavailability and pharmacokinetic properties of two different enteric formulations of esomeprazole in healthy bangladeshi male volunteers: An open-label, single-dose, randomized-sequence, two-way crossover study. Clin Ther. 2010; 32(7):1419–426.

11. Li X-Q, Weidolf L, Simonsson R, Andersson TB. Enantiomer/enantiomer interactions between the S-and R-isomers of omeprazole in human cytochrome P450 enzymes: major role of CYP2C19 and CYP3A4. J Pharmacol Exp Ther. 2005; 315(2):777–787.

12. Äbelö A, Andersson TB, Bredberg U, Skånberg I, Weidolf L. Stereoselective metabolism by human liver CYP enzymes of a substituted benzimidazole. Drug Metab Dispos. 2000; 28(1):58–64.

13. Andersson T, Miners JO, Veronese ME, Tassaneeyakul W, Meyers URSA, et al. Identification of human liver cytochrome P450 isoforms mediating omeprazole metabolism. Br J Clin Pharmacol. 1993; 36(6):521–530.

14. Hultman I, Stenhoff H, Liljeblad M. Determination of esomeprazole and its two main metabolites in human, rat and dog plasma by liquid chromatography with tandem mass spectrometry. J Chromatogr B Anal Technol Biomed Life Sci. 2007; 848(2):317–322.

15. Mogili R, Bannoth K, Chandu BR, Challa BR. Quantification of esomeprazole in human plasma by liquid chromatography tandem mass spectrometry and its application to bioequivalence study. Der Pharm Lett. 2011; 3:138–145.

16. Gopinath S, Kumar RS, Shankar M bhawani, Danabal P. Development and validation of a sensitive and high-throughput LC-MS/MS method for the simultaneous determination of esomeprazole and naproxen in human plasma. Biomed Chromatogr. 2013; 27(7):894–899.

17. Validation BM. Guidance for Industry Bioanalytical Method Validation Guidance for Industry Bioanalytical Method Validation. FDA 2018;(May):1–22.

18. Cluver CA, Hannan NJ, van Papendorp E, Hiscock R, Beard S, Mol BW, et al. Esomeprazole to treat women with preterm preeclampsia: a randomised placebo controlled trial. Am J Obstet Gynecol. 2018; 219(4):388.e1-388.e17.

19. Cluver CA, Walker SP, Mol BW, Theron GB, Hall DR, Hiscock R, et al. Double blind, randomised, placebocontrolled trial to evaluate the efficacy of esomeprazole to treat early onset pre-eclampsia (PIE Trial): A study protocol. BMJ Open. 2015;

5(10):1–8.

20. Nexium (esomeprazole magnesium) delayed-release capsules. Wilmington, DE. AstraZeneca, LP. 2006. Availabe from: https//www.accessdata.fda.gov/drugsatfda_docs/label/2006/021153s022lbl.pdf.

OBSTETRICS

Esomeprazole to treat women with preterm preeclampsia: a randomized placebo controlled trial

Catherine A. Cluver, MMed; Natalie J. Hannan, PhD; Erika van Papendorp, BN; Richard Hiscock, MBBS; Sally Beard, BSc; Ben W. Mol, PhD; Gerhard B. Theron, MD; David R. Hall, MD; Eric H. Decloedt, MMed; Marietjie Stander, PhD; Kim T. Adams, BSc; Megan Rensburg, MMed; Pawel Schubert, MPhil; Susan P. Walker, MD; Stephen Tong, PhD

BACKGROUND: Preterm preeclampsia has a high rate of fetal death or disability. There is no treatment to slow the disease, except delivery. Preclinical studies have identified proton pump inhibitors as a possible treatment.

OBJECTIVE: The purpose of this study was to examine whether esomeprazole could prolong pregnancy in women who have received a diagnosis of preterm preeclampsia.

STUDY DESIGN: We performed a double-blind, randomized controlled trial at Tygerberg Hospital in South Africa. Women with preterm preeclampsia (gestational age 26 weeks+0 days to 31 weeks+6 days) were assigned randomly to 40-mg daily esomeprazole or placebo. The primary outcome was a prolongation of gestation of 5 days. Secondary outcomes were maternal and neonatal outcomes. We compared circulating markers of endothelial dysfunction that was associated with preeclampsia and performed pharmacokinetic studies.

RESULTS: Between January 2016 and April 2017, we recruited 120 participants. One participant was excluded because of incorrect randomization, which left 59 participants in the esomeprazole and 60 participants in the placebo group. Median gestational age at enrolment was 29+4 weeks gestation. There were no between-group differences in median time from randomization to delivery: 11.4 days (interquartile range, 3.6−19.7 days) in the esomeprazole group and 8.3 days (interquartile range, 3.8−19.6 days) in the placebo group (3 days longer in the esomeprazole arm; 95% confidence interval, −2.9−8.8; P=.31). There were no placental abruptions in the esomeprazole group and 6 (10%) in the placebo group (P=.01, P=.14 adjusted). There were no differences in other maternal or neonatal outcomes or markers of endothelial dysfunction. Esomeprazole and its metabolites were detected in maternal blood among those treated with esomeprazole, but only trace amounts in the umbilical cord blood.

CONCLUSION: Daily esomeprazole (40 mg) did not prolong gestation in pregnancies with preterm preeclampsia or decrease circulating soluble fms-like tyrosine kinase 1 concentrations. Higher levels in the maternal circulation may be needed for clinical effect.

Key words: esomeprazole, trial, preterm preeclampsia, sFlt1, pharmacokinetics

Preeclampsia is one of the most serious complications of pregnancy. It affects 3−8 % of pregnancies and is a leading cause of maternal, fetal, and neonatal morbidity.[1,2] There is no treatment that can slow disease progression, and the only treatment option is to deliver the pregnancy. For preeclampsia that occurs at preterm gestations, clinicians are often required to deliver the fetus early, which results in iatrogenic prematurity with a risk of major disability that includes cerebral palsy, intracerebral bleeding, retinopathy of prematurity, chronic lung disease, and death. The risks of these complications are higher if pregnancies are delivered at earlier gestations.[3] If a treatment were available that temporizes disease progression, it could be used to safely delay delivery to gain gestation, thereby decreasing the degree of prematurity and improving perinatal outcomes.

The preeclamptic placenta releases elevated levels of soluble fms-like tyrosine kinase 1 (sFlt1) and soluble endoglin into the maternal circulation.[4] These antiangiogenic factors cause maternal endothelial dysfunction, hypertension, and multiorgan injury.[5] Esomeprazole is a proton pump inhibitor (PPI) that is prescribed widely in pregnancy to relieve symptomatic gastric reflux. Members of our team have performed preclinical laboratory studies that have shown that PPIs such as esomeprazole are a candidate therapeutic for preeclampsia.[6] Esomeprazole, in particular, has been shown to have diverse biologic actions. Firstly esomeprazole decreases sFlt1 and soluble endoglin production and release from primary trophoblast cells and placental tissue explants and primary endothelial cells/tissues in both normal and preeclamptic pregnancies. Secondly esomeprazole was able to dilate whole human vessels from both normal pregnancies treated with a constrictor and vessels that were obtained from women with preeclampsia. Thirdly, preclinical studies also showed that esomeprazole decreased endothelial dysfunction by mitigating tumor necrosis α−induced endothelial injury, as demonstrated by reducing expression of endothelial vascular cell adhesion molecule-1 and reduced leucocyte adhesion to the endothelium. Lastly important animal studies clearly show that esomeprazole reduces blood pressure in a transgenic mouse model of preeclampsia in which human sFlt1 is overexpressed in the placenta and released in excess into the maternal blood, as seen in women with preeclampsia.[6] Others have subsequently found decreased circulating sFlt1 and soluble endoglin levels in an existing cohort of bloods of women with suspected or confirmed preeclampsia that were coincidentally taking PPIs.[7]

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AJOG at a Glance

Why was this study conducted?

Preeclampsia has high rates of fetal death or disability. There is no treatment to slow the disease, except delivery. Preclinical studies have identified proton pump inhibitors as a possible treatment.

Key findings

Daily oral esomeprazole (40 mg) did not prolong gestation in pregnancies with preterm preeclampsia or decrease circulating soluble fms-like tyrosine kinase 1 concentrations.

What does this add to what is known?

This is the first trial for preterm preeclampsia that has integrated clinical outcomes, mechanistic studies, and pharmacokinetics. Oral esomeprazole (40 mg) may be too low a dose to treat preterm preeclampsia; higher doses may still be effective. This may be the fastest completed randomized clinical trial of a treatment for preterm preeclampsia. It is possible to complete clinical trials for preterm preeclampsia in a reasonable timeframe by running the trials in settings in which the incidence of disease is high.

These promising preclinical data suggest that esomeprazole is a potential candidate treatment; we therefore set out to examine whether oral esomeprazole may be an effective treatment for preterm preeclampsia.

Methods

Trial design

In this single-site phase II double-blind, randomized, placebo-controlled clinical trial, we compared oral esomeprazole with placebo. A 40 mg daily dose was selected based on pharmacokinetic data that showed effective suppression of gastrointestinal symptoms in nonpregnant patients and on reassuring data that showed no adverse effects if taken during pregnancy.[8-11] The trial site was Tygerberg Hospital, Cape Town, South Africa, which is a large academic referral center that is situated in a region with high rates of preeclampsia. We have published the protocol,[12] and the trial was registered with the Pan African Clinical Trials Registry (PACTR201 504000771349).

Pregnant women with singleton pregnancies were invited to participate if they had been diagnosed with preterm preeclampsia between 26+0 and 31+6 weeks gestation. The gestation at enrolment was determined by either menstrual dates (if the women was certain of her last menstrual period) or by an early or mid-trimester pregnancy ultrasound examination. Both the managing perinatologist and neonatologist had to agree that expectant management could benefit the fetus.

Women were not eligible if they had an indication for immediate delivery because they could not be treated expectantly to gain further fetal maturity. Exclusion criteria therefore included established maternal or fetal compromise that necessitated delivery, the current use or contraindications to the use of PPIs, and the use of medications that could interact with PPIs (which included warfarin, ketoconazole, voriconazole, atazanavir, nelfinavir, saquinavir, digoxin, St John's Wort, rifampin, cilostazol, diazepam, tacrolimus, erlotinib, methotrexate, and clopidogrel). Specific clinical exclusion criteria included eclampsia, severe hypertension not be controlled within 48 hours of admission, a cerebrovascular event, posterior reversible encephalopathy syndrome, severe renal impairment with a creatinine >125 μmol/L, pulmonary edema, disseminated intravascular coagulation, hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, liver hematoma or rupture, severe ascites on ultrasound examination. We excluded pregnancies with a suspicion of a major fetal anomaly or malformation. Expectant management involved hospital admission with close maternal and fetal surveillance. Maternal surveillance involved 4 hourly blood pressure measurement, twice daily clinical assessments, daily urinalysis, and twice weekly biochemical testing. Fetal surveillance involved 6 hourly cardiotocography and ultrasound assessments every 2 weeks or more frequently, if indicated. To enhance fetal lung maturity, all participants received 2 doses of betamethasone that were given 24 hours apart, followed by a single repeat dose 1 week later if not delivered, as per local protocol.[13] Expectant management ended at 34 weeks gestation; women who reached this gestation were delivered. Delivery at <34 weeks gestation was a clinical decision made by the patient's treating team.

The study participants provided written informed consent. The study had Health Research Ethics Committee (HREC) approval, was approved by the South African Medicines Control Council. Study data were collected and managed with the use of REDCap electronic data capture tools.[14]

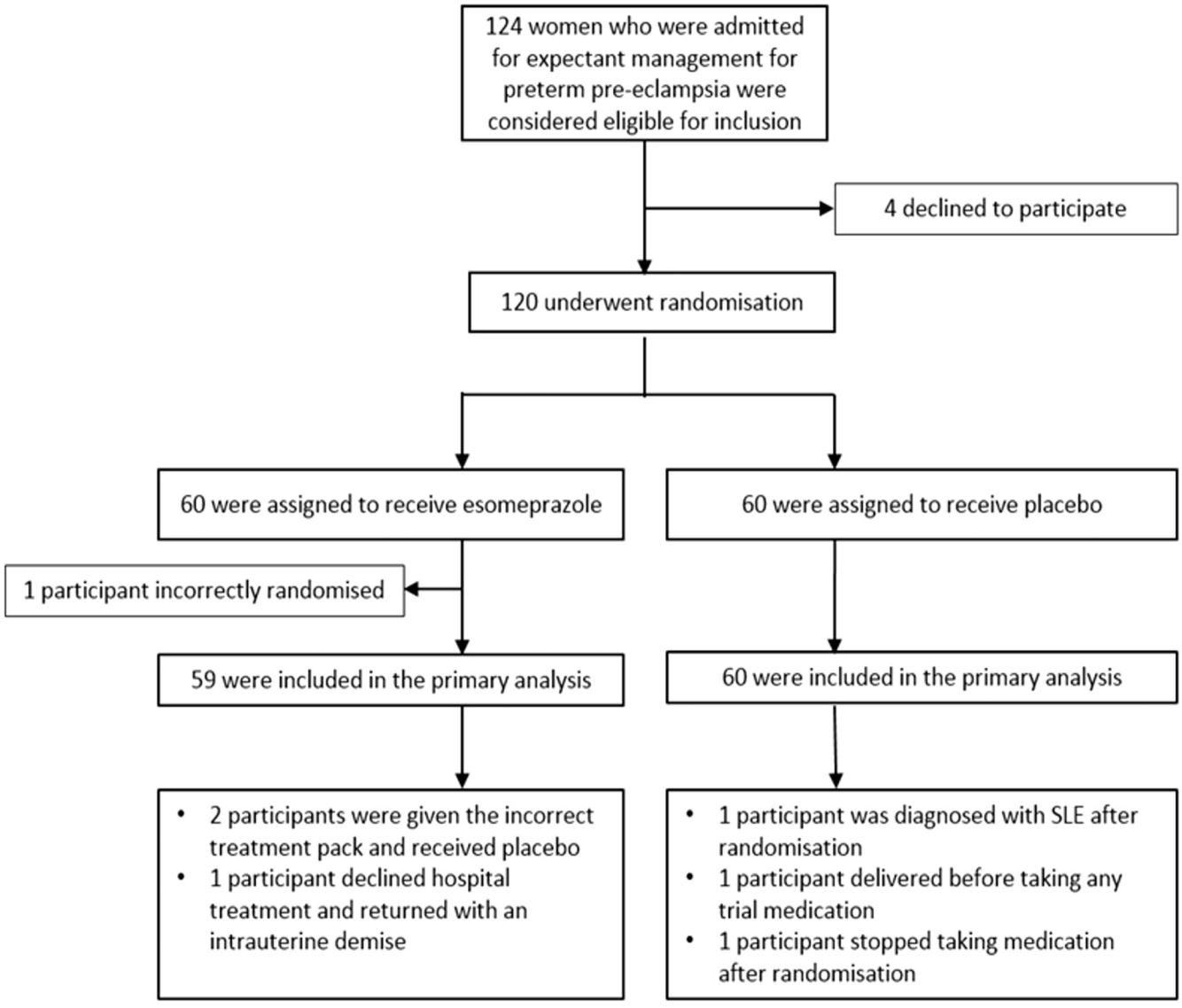
Randomization and masking

Randomization was performed in a 1:1 ratio with the use of an online, web-based sequence generator. Because gestation at randomization could possibly impact the length of pregnancy prolongation, randomization was stratified (strata 1 was ≤28+6 weeks; strata 2 was 29+0 until 31+6 weeks gestation). Randomization was done within blocks of random size within 4−6. The tablets and treatment packs were manufactured, packed, and labelled by the Institute of Drug Technology Limited (en.idtaus.com.au) in Victoria, Australia, and were identical with respect to variables such as size, thickness, physical properties, and appearance. The investigators had no access to the randomization list, and allocation concealment was maintained throughout the trial.

Placental and blood collection to measure angiogenic markers of preeclampsia and endothelial dysfunction and to perform pharmacokinetics

Plasma samples to measure circulating preeclampsia and angiogenic biomarkers were collected at randomization and twice weekly until delivery. Placental tissue

FIGURE 1
Flowchart of screening, randomization, and follow up



The flowchart summarizes the screening, randomization, allocation to esomeprazole or placebo, exclusion after randomization, complications and follow up of the study.

SLE, systemic lupus erythematosus.

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samples and umbilical artery cord blood were collected at delivery when possible. After recruitment was completed, circulating concentrations and placental expression of molecules that are markers of preeclampsia and endothelial dysfunction were measured.

Pharmacokinetics was performed in a subgroup of patients who had been administered esomeprazole. Plasma samples were drawn from a catheter in a forearm vein at the following dosing interval: predose, at 15, 30, and 45 minutes; postdose, at 1, 1.5, 2, 4, 8, and 24 hours. Levels were measured in batch after the trial was completed (the Supplemental Material provides further details on how the esomeprazole was measured).

Outcome measures

The primary outcome was prolongation of pregnancy, and the study was powered to show a prolongation of 5 days. Secondary outcomes included composite and individual maternal, fetal, and neonatal outcomes, maternal biomarkers, pharmacokinetics, and placental samples.

After completion of the trial, we measured the plasma circulating concentrations of the following markers of preeclampsia: sFlt1, soluble endoglin, placental growth factor (PlGF) with the

TABLE 1
Characteristics of trial participants at enrolment

Characteristics	Esomeprazole (n=59)	Placebo (n=60)
Gestation at randomization, wk+d		
Median [interquartile range]	29+4 [27+6–30+6]	29+5 [28+1–30+5]
Mean (standard deviation)	29.4 (1.65)	29.4 (1.66)
Gestation <29 weeks at randomization, n (%)[a]	20 (33.9)	20 (33.3)
Maternal age (y), median [interquartile range]	24 [21–31]	30 [25–34]
Body mass index (kg/m^2), median [interquartile range]	29.4 [24.8–33.3]	29.0 [24.0–35.2]
Race or ethnicity, n (%)		
Black	34 (57.6)	33 (55)
Colored (multiracial ethnic group native to Southern Africa)	25 (42.4)	27 (45.0)
Smoking, n (%)	8 (13.6)	4 (6.7)
Aspirin use, n (%)	1 (1.7)	0
Calcium use, n (%)	1 (1.7)	0
HIV positive, n (%)	8 (13.6)	12 (20.0)
Chronic hypertension, n (%)	13 (22.0)	21 (35.0)
Nulliparous, n (%)	26 (44.1)	12 (20)
Multiparous, n (%)		
Without hypertension in a previous pregnancy	25 (42.4)	27 (45)
With hypertension in a previous pregnancy	8 (13.6)	21 (34.9)
New paternity in current pregnancy, n (%)	11/37 (29.7)	17/48 (35.4)
Highest systolic blood pressure before randomization (mm Hg), mean (standard deviation)	166 (17.5)	168 (16.4)
Highest diastolic blood pressure before randomization (mm Hg), mean (standard deviation)	103 (13.4)	103 (11.4)
24-Hour protein creatinine ratio at enrolment (g/24 hr), median [interquartile range]	1.46 [0.62-3.16]	1.06 [0.57-16.86]
Hemoglobin (g/dL), mean (standard deviation)	12.3 (1.5)	11.6 (1.4)
Platelet count (10^9/L), mean (standard deviation)	207 (59.9)	222 (67.2)
Urea (mmol/L), mean (standard deviation)	4.0 (1.64)	3.7 (1.4)
Creatinine (mg/dL), mean (standard deviation)	0.05 (0.015)	0.05 (0.013)
Estimated fetal weight (g), mean (standard deviation)	1153 (300.4)	1153 (217.7)
Fetal weight percentile, median [interquartile range]	6.0 [2.1-24.8]	9.5 [1.7-22.5]
Absent blood flow on umbilical artery Doppler, n (%)	2 (3.4)	4 (6.7)

[a] Percentage of each group.

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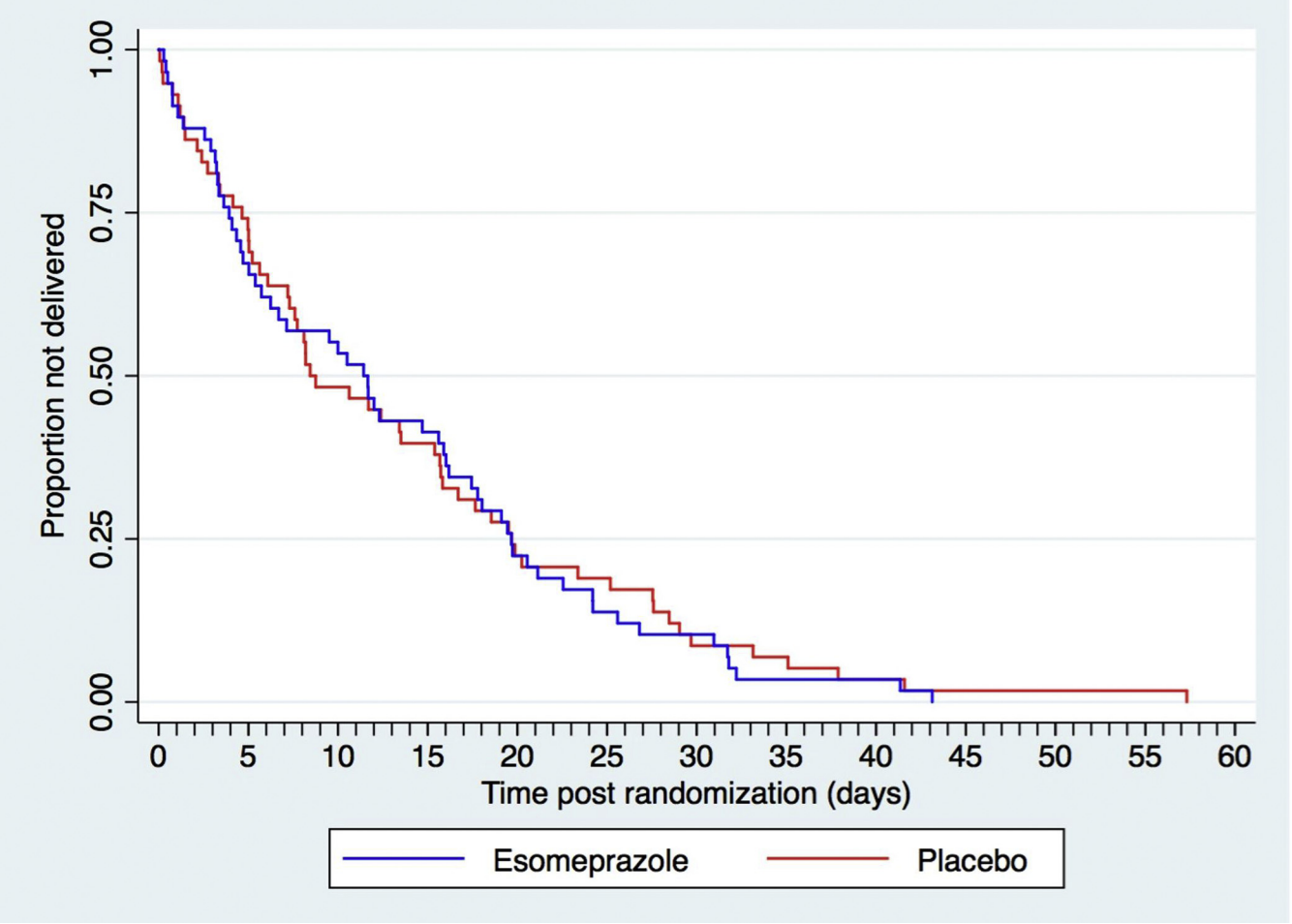
use of commercially available enzyme-linked immunosorbent assays. We also measured markers of endothelial dysfunction: endothelin-1, vascular endothelial cell adhesion molecule-1 (VCAM-1).

Total RNA was extracted from the placental biopsy specimens that were collected at delivery; the expression of sFlt1, PlGF, vascular endothelial growth factor-1, and the anti-oxidant molecule heme oxygenase-1 was measured by polymerase chain reaction (Supplemental Material).

Adherence and adverse events

Medication adherence was checked daily. After delivery, the treatment packs were collected, and the remaining tablets were counted. The trial midwife reviewed participants daily for adverse events. Serious adverse events were reported to the Data Monitoring and Safety Committee and Health Research Ethics Committee and were handled in accordance with Good Clinical Practice guidelines.

FIGURE 2
Survival curve



Survival curve shows the proportion of trial participants who remained undelivered, graphed against the number of days of gestation after randomization. *Blue* indicates the women who were treated with esomeprazole; *red* indicates the women who were treated with placebo.

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Statistical analysis

The sample size was based on data on the duration of expectant management at Tygerberg Hospital.[15] To identify a gain in gestation of 5 days, we needed to recruit 86 women (90% power, 2-sided alpha 0.05). This sample size was multiplied by 1.15 to statistically correct for non-normality. An additional 10 per arm were added to account for anticipated drop-outs. Thus, a total of 120 participants (60 per arm) had to be recruited.

Statistical analyses were performed on an intention-to-treat principle. A 2-sided *P*-value $<.05$ was considered to indicate statistical significance. The primary outcome was tested with the use of quantile regression analysis with the treatment group and gestational strata as covariates. Results are presented as median group difference with 95% confidence interval (95% CI). Survival analyses were done with Cox proportional hazards regression and graphed with Kaplan-Meier survivorship curves. Continuous variables were compared with either *t*-test (normally distributed variables) or Mann-Whitney *U* (nonnormally distributed data). Categoric values were compared with the use of the Fisher's exact test.

For circulating biomarker studies, between-group comparisons of circulating analyte concentrations were performed by a marginal mean model that was estimated with the use of generalized estimating equations to allow for both within patient correlation and missing samples. Graphic presentation used median, 25th, and 75th percentiles that were calculated from samples that were available at each day after random assignment. A smoothed scatterplot of these quantiles was constructed with the use of kernel-weighted local polynomial regression over a prespecified number of time units each side of the time of interest. The analysis used an Epanechnikov kernel function, automatic optimization of the degree of polynomials, and a bandwidth of 4 days.

Results

Trial participants

Participants were recruited from January 2016 until April 2017

TABLE 2
Outcomes according to trial group

Outcome	Esomeprazole (n=59)	Placebo (n=60)	*P* value
Primary			
Prolongation of gestation, d			
Median [interquartile range]	11.4 [3.6–19.7]	8.3 [3.8–19.6]	.31
Mean (standard deviation)	12.9 (10.8)	13.1(12.2)	
Gestation at delivery (wk+d), median [interquartile range]	31+2 [29+3–33+3]	31+3 [29+3–33+4]	.93
Secondary			
Composite maternal outcome, n (%)[a]	1 (1.7)	4 (6.7)	.36
Individual maternal outcomes			
Eclampsia, n (%)	0	3 (5.0)	.24
Pulmonary edema, n (%)	1 (1.7)	1 (1.7)	.99
Admission to high care unit or intensive care unit, n (%)	3 (5.1)	6 (10.0)	.49
Proteinuria ≥3g/24h, n (%)	22 (37.3)	24 (40)	.85
Systolic blood pressure >160 mm Hg, n (%)	29 (49.2)	24 (40.0)	.36
Diastolic blood pressure >110 mm Hg, n (%)	13 (22.0)	8 (13.3)	.24
Highest systolic blood pressure during trial (mm Hg), mean (standard deviation)	160 (11.9)	160 (12.3)	.91
Highest diastolic blood pressure during trial (mm Hg), mean (standard deviation)	102 (10.6)	101 (8.7)	.57
Platelet count <50×109, n (%)	0	1 (1.7)	.99
HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome, n (%)	5 (8.5)	3 (5.0)	.49
Aspartate aminotransferase (level) >60 μ/L, n (%)	3 (5.1)	1 (1.7)	.30
Hemolysis (lactate dehydrogenase >600 μ/L) or hemolysis on peripheral blood smear or decreased haptoglobin, n (%)	2 (3.4)	3 (5.0)	.99
Placental abruption, n (%)	0	6 (10.0)	.03
Major postpartum hemorrhage, n (%)	0	3 (5.0)	.24
Thromboembolic disease, n (%)	1 (1.7)	0	.99
Moderate-to-severe ascites, n (%)	7 (11.9)	4 (6.7)	.36
Composite fetal outcome, n (%)[b]	49 (83.1)	45 (75)	.37
Individual fetal outcomes			
Persistent absent flow in umbilical artery Doppler, n (%)	4 (6.8)	7 (11.7)	.53
Redistribution in the middle cerebral artery, n (%)	28 (47.5)	27 (45)	.85
Growth restriction (estimated fetal weight <10th percentile), n (%)	38 (64.4)	30 (50)	.14
Significant changes in fetal heart rate pattern necessitating delivery, n (%)	28 (47.5)	26 (43.3)	.74
Intrauterine death, n (%)	1 (1.7)	1 (1.7)	.99
Neonatal composite outcome, n (%)[c]	10 (16.9)	11 (18.3)	.88

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(continued)

(Figure 1). Of 124 women who were admitted with preterm preeclampsia who were considered eligible, 4 women declined to participate (96.7% recruitment rate). One participant in the esomeprazole group was excluded after randomization because it was later discovered that she did not meet the trial criteria for a diagnosis of preeclampsia because she did not have significant hypertension and proteinuria. This left 59 women in the esomeprazole group. Two participants in this group were given the incorrect treatment pack and received placebo.

TABLE 2
Outcomes according to trial group *(continued)*

Outcome	Esomeprazole (n=59)	Placebo (n=60)	*P* value
Individual neonatal outcomes			
Neonatal death within 6 weeks after the due date, n (%)	7 (11.9)	9 (15.0)	.67
Grade III or IV intraventricular hemorrhage, n (%)	2 (3.4)	0	.24
Necrotizing enterocolitis, n (%)	4 (6.8)	3 (5.0)	.72
Bronchopulmonary dysplasia, n (%)	1 (1.7)	0	.50
Apgar score <7 at 5 minutes, n (%)	1 (1.7)	7 (11.7)	.06
Umbilical artery pH <7.05, n (%)	1/35 (2.9)	2/34 (5.9)	.61
Surfactant use, n (%)	14 (23.7)	9 (15.0)	.25
Neonatal intensive care unit admission, n (%)	8 (13.6)	4 (6.7)	.24
High care unit admission, n (%)	53 (89.8)	45 (75.0)	.05
Intubation and mechanical ventilation, n (%)	6 (10.2)	6 (10.0)	.99
Continuous positive airway pressure support, n (%)	46 (78.0)	39 (65.0)	.16
Grade III or IV hyaline membrane disease, n (%)	7 (11.9)	9 (15.0)	.79
Retinopathy of prematurity, n (%)	2 (3.4)	0	.24
Neonatal sepsis, n (%)	9 (15.3)	5 (8.3)	.27
Birthweight (g), mean (standard deviation)	1343 (466.5)	1379 (441.3)	.54
Discharge time (d), median [interquartile range]	3 (3–5)	3 (3–4)	.24

NOTE: No participant had any of the following outcomes: maternal death, severe renal impairment, cerebral vascular event, liver hematoma or rupture, posterior reversible encephalopathy syndrome, left ventricular failure, serum creatinine >125 μmol, disseminated intravascular coagulation, home oxygen support, persistent reversed flow in the umbilical artery Doppler.

[a] Included the occurrence of any of the following serious maternal outcomes: maternal death, eclampsia, pulmonary edema (oxygen saturation ≤90%, with clinical signs and symptoms that required treatment), severe renal impairment or the need for dialysis, a cerebral vascular event, and liver hematoma or rupture; [b] Reversed a-wave in the ductus venosus, significant changes in fetal heart rate pattern that necessitated delivery, intrauterine fetal death, fetal growth restriction, persistent reversed flow in the umbilical artery, redistribution in the middle cerebral artery Doppler, reversed a-wave in the ductus venosus Doppler; [c] Neonatal death within 6 weeks after the expected due date, grade III or IV intraventricular hemorrhage, necrotizing enterocolitis; and bronchopulmonary dysplasia.

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One participant in this group declined hospital treatment 1 week after randomization, left the hospital, and returned with a stillbirth. Sixty women were allocated to placebo, and all were included in the analysis. One participant delivered before taking her trial medication, and 1 participant was diagnosed with systemic lupus erythematosus after randomization. One participant in this group stopped taking her medication a few days before delivery. The maternal characteristics and obstetrics history of the cohort are shown in Table 1.

The median gestational age at randomization was 29 weeks 4 days in the esomeprazole group and 29 weeks 5 days in the placebo group. The placebo group had a higher median maternal age at enrolment. There were also more multiparous women, women with underlying hypertension, and women who had a previous pregnancy complicated by hypertension in the placebo group.

Primary outcome

The median time from randomization to delivery was 11.4 days (mean, 12.9 days) in the esomeprazole group vs 8.3 days (mean, 13.1 days) in the placebo group. There was no significant difference in median prolongation between treatment groups either unadjusted (median difference, 3.0; 95% CI, −2.9 to 8.8; *P*=.31) or adjusted for gestational age strata (median difference, 0.81; 95% CI, −5.1 to 6.7; *P*=.79). There was also no difference in the median prolongation between strata when adjusted for treatment group (median difference, 3.0; 95% CI, −3.2 to 9.2; *P*=.34) days.

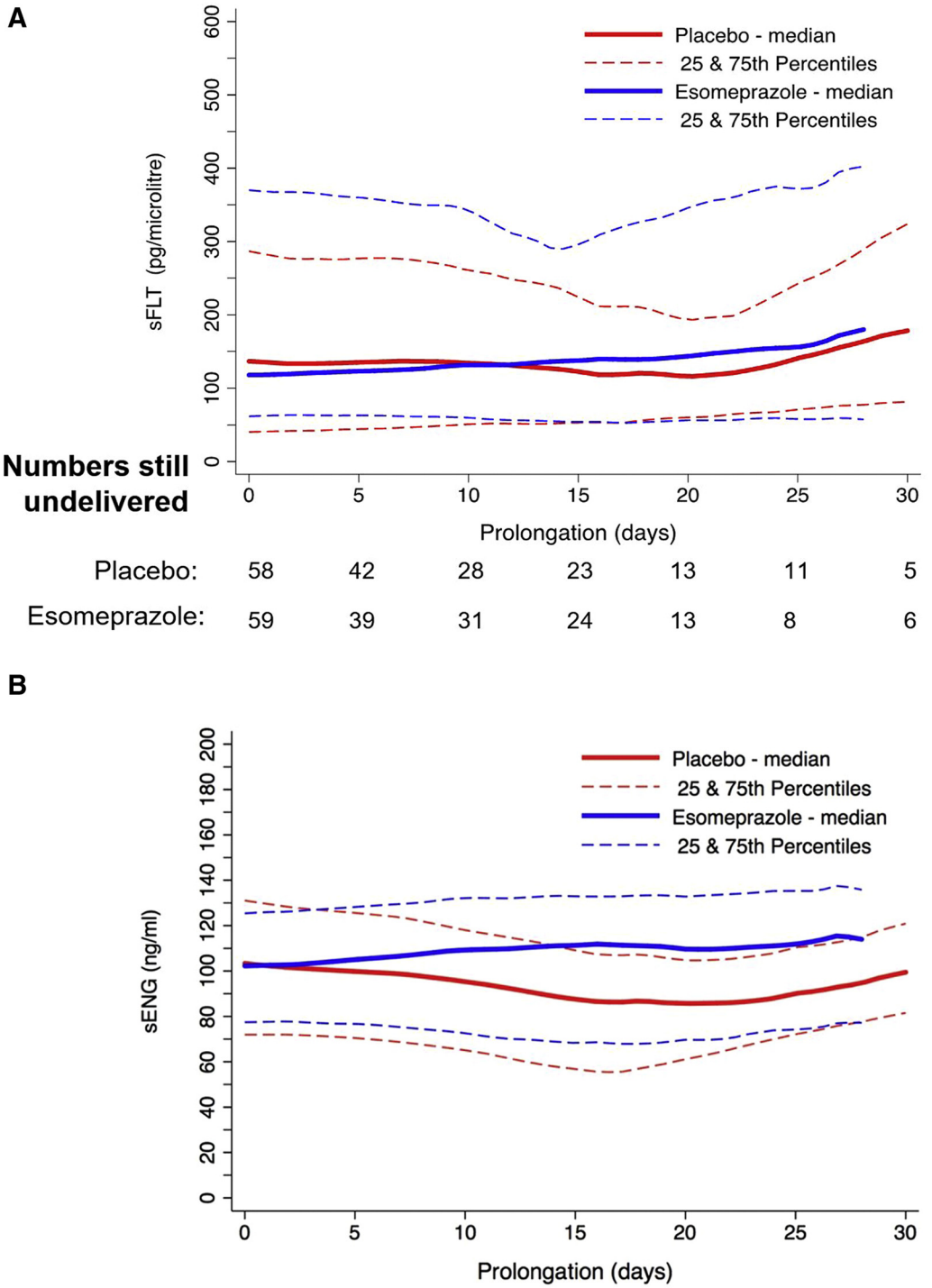
There was no difference in the instantaneous hazard of delivery, at any time, between the 2 treatment arms for either stratum (Figure 2). The estimated hazard ratio was 1.13 (95% CI, 0.70−2.17; *P*=.70) for <29 weeks and 1.07 (95% CI, 0.68−1.68; *P*=.78) for ≥29 weeks.

Secondary outcomes

There were no significant differences between treatment groups for any of the maternal, fetal, and neonatal composite or individual outcomes (Table 2), except for placental abruption. There were no placental abruptions (0/59) in the esomeprazole group and 10% (6/60) in the placebo group (*P*=.01), which was not significant when we adjusted for the fact that we performed multiple comparisons for other secondary outcomes (*P*=.14).

SFlt1 and soluble endoglin are antiangiogenic factors that are increased

FIGURE 3
Circulating plasma levels of antiangiogenic factors in women who were treated with either placebo or esomeprazole



A, Median circulating plasma soluble fms-like tyrosine kinase 1 concentrations (*solid lines*) and 25th and 75th percentiles (*dotted lines*) among participants administered placebo (*red*) or esomeprazole (*blue*). There were no differences in circulating soluble fms-like tyrosine kinase 1 levels between groups. **B,** Median circulating plasma soluble endoglin concentrations (*solid line*), and 25th and 75th percentiles (*dotted line*) among participants administered placebo (*red*) or esomeprazole (*blue*). There were no differences in circulating soluble endoglin levels between groups. Numbers that were still undelivered at each 5-day time point and that could have contributed to the data for soluble fms-like tyrosine kinase 1 or soluble endoglin are shown in **A.**

sENG, soluble endoglin; *sFlt1*, soluble fms-like tyrosine kinase 1.

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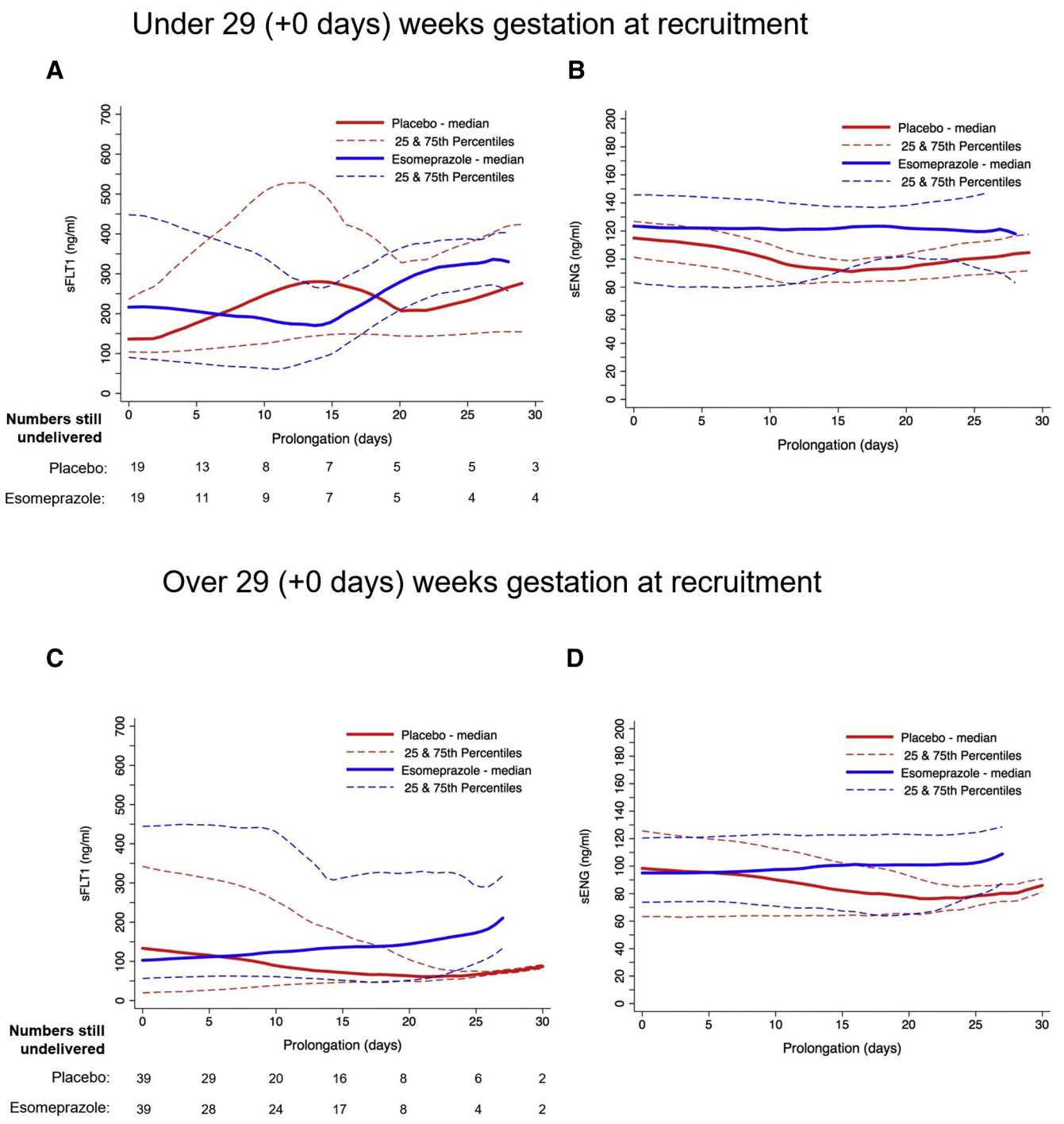
FIGURE 4
Circulating plasma levels of antiangiogenic factors in women who were treated with either placebo or esomeprazole



The graphs show analyte concentrations among the subcohorts of women who were either under (**A** and **B**) or over (**C** and **D**) 29 weeks gestation at recruitment. All graphs depict median circulating plasma concentrations (*solid lines*) of analytes and the 25th and 75th percentiles (*dotted lines*). None of the comparisons between esomeprazole (*blue*) and placebo (*red*) were significant. Numbers that were still undelivered at each 5-day time point and that could have contributed to the data are shown in **A** and **C** for soluble fms-like tyrosine kinase 1. The numbers that were left undelivered for soluble endoglin for **B** are the same as that shown in **A** for soluble fms-like tyrosine kinase 1; and the numbers that were left undelivered for **D** are the same as that shown in **C** for soluble fms-like tyrosine kinase 1.

sENG, soluble endoglin; *sFlt1*, soluble fms-like tyrosine kinase 1.

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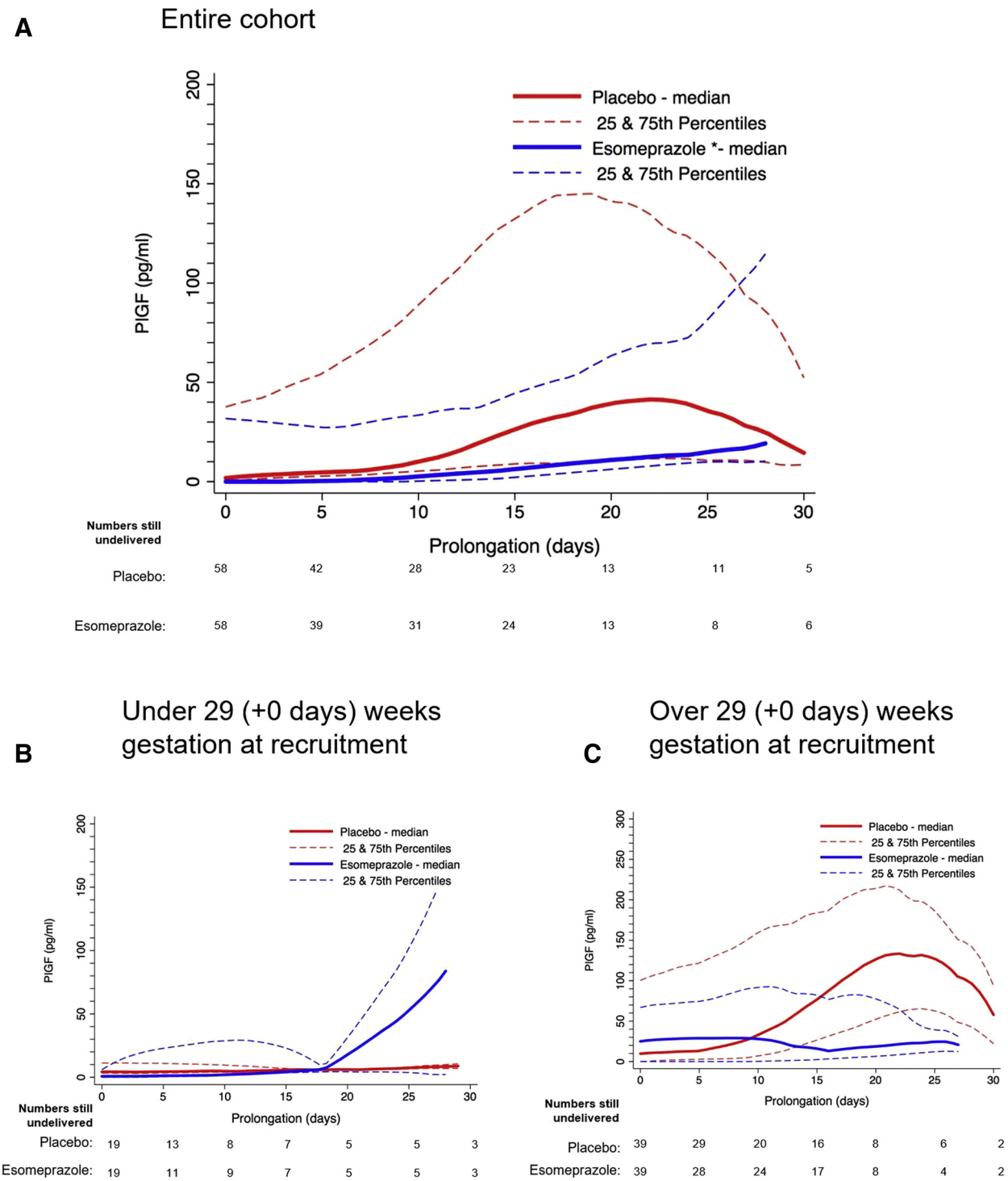
FIGURE 5
Circulating plasma levels of placental growth factor in women who were treated with either placebo or esomeprazole



The graphs show analyte for **A,** the entire cohort or the entire cohort split according to whether women were **B,** under or **C,** over 29 weeks gestation at recruitment. All graphs depict median circulating plasma concentrations (*solid lines*) of analytes and the 25th and 75th percentiles (*dotted lines*). None of the comparisons between esomeprazole (*blue*) and placebo (*red*) were significant. Numbers that were still undelivered at each 5-day time point and could have contributed to the data are shown below each graph.

PlGF, placental growth factor.

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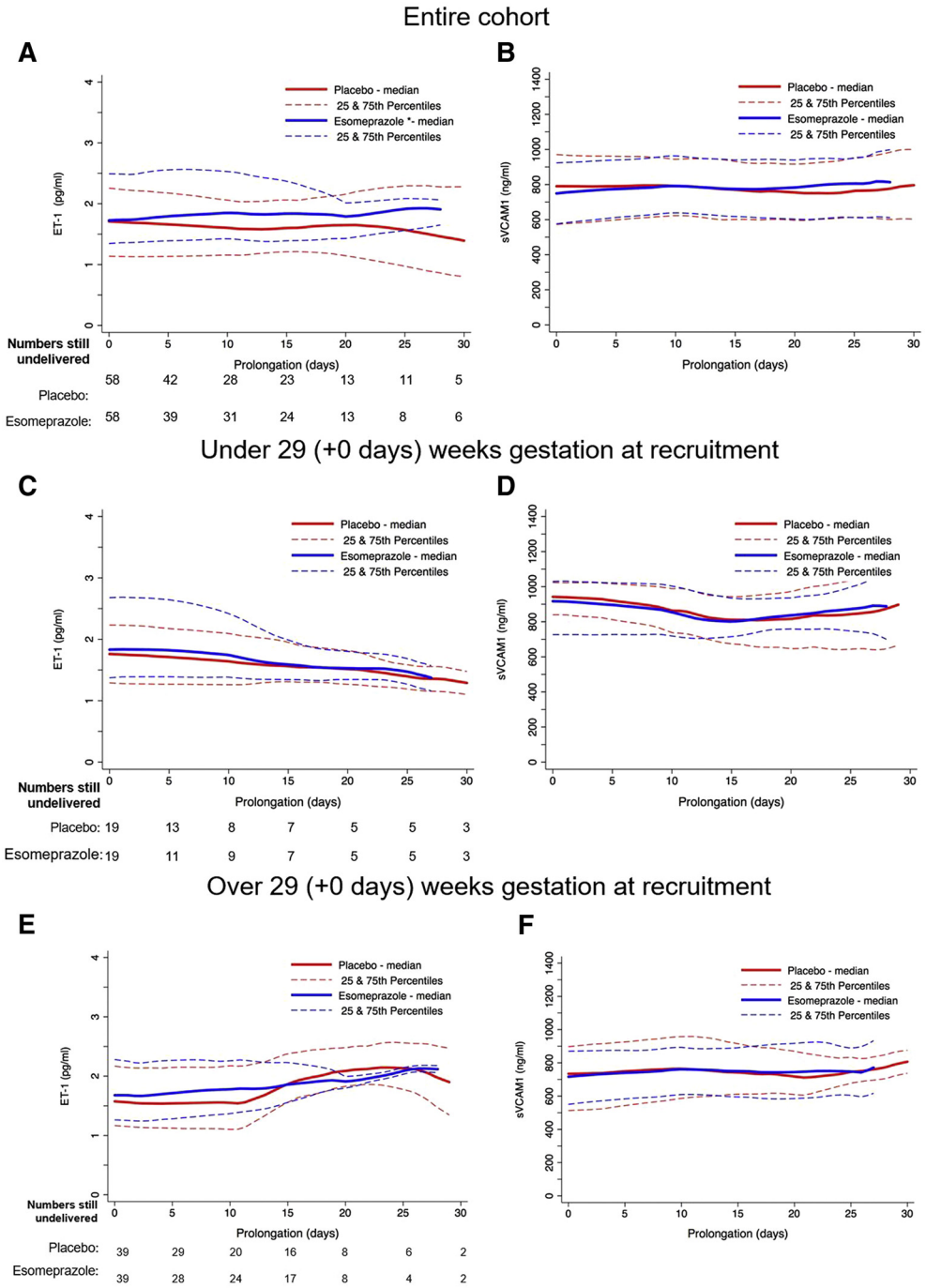
FIGURE 6
Circulating plasma levels of endothelin1 and soluble vascular cell adhesion molecule-1 in women who were treated with either placebo or esomeprazole



The graphs show analyte concentrations among **A** and **B,** the entire cohort or the entire cohort split according to whether women were **C** and **D,** under or **E** and **F,** over 29 weeks gestation at recruitment. All graphs depict median circulating plasma concentrations (*solid lines*) of analytes, and the 25th and 75th centiles (dotted lines). None of the comparisons between esomeprazole (*blue*) and placebo (*red*) were significant. Numbers that were still undelivered at each 5-day time point and that could have contributed to the data for **A** and **B** are shown below graph **A**; numbers that were still undelivered at each 5-day time point and that could have contributed to the data for **C** and **D** are shown below graph **C**; numbers that were still undelivered at each 5-day time point and that could have contributed to the data for **E** and **F** are shown below graph **E**.

ET-1, endothelin -1; *sVCAM1*, soluble vascular cell adhesion molecule-1.

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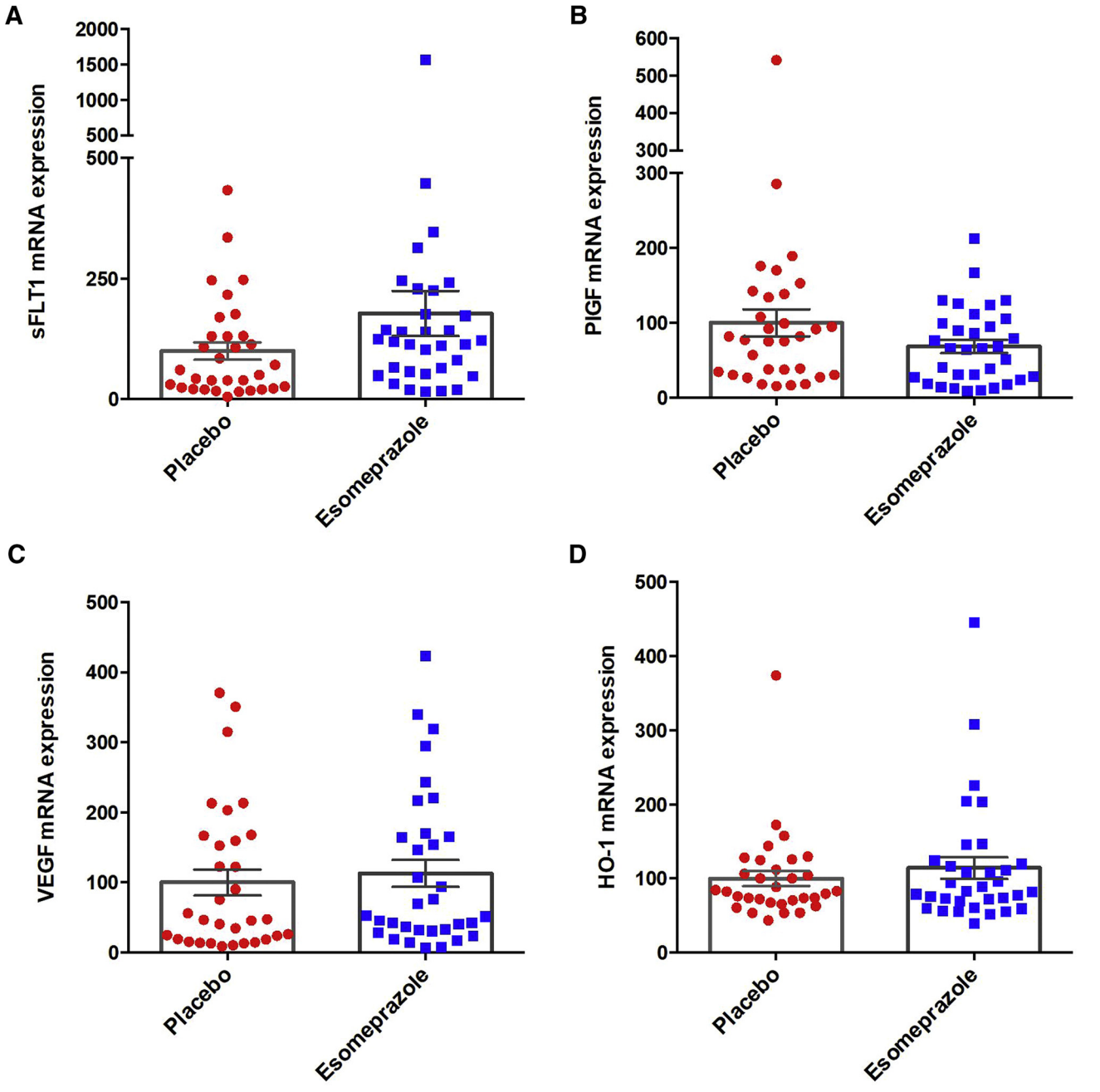
FIGURE 7
Placental messenger RNA expression



A, Soluble fms-like tyrosine kinase 1, **B,** placental growth factor, **C,** vascular endothelial growth factor, and **D,** heme oxygenase -1 in placental tissues that were collected from women who received placebo (n=32) or esomeprazole (n=33). None of the comparisons were significant. Data are mean fold change±standard error of the mean.

HO-1, heme oxygenase-1; *mRNA*, messenger RNA; *PlGF*, placental growth factor; *sFlt1*, soluble fms-like tyrosine kinase 1; *VEGF*, vascular endothelial growth factor.

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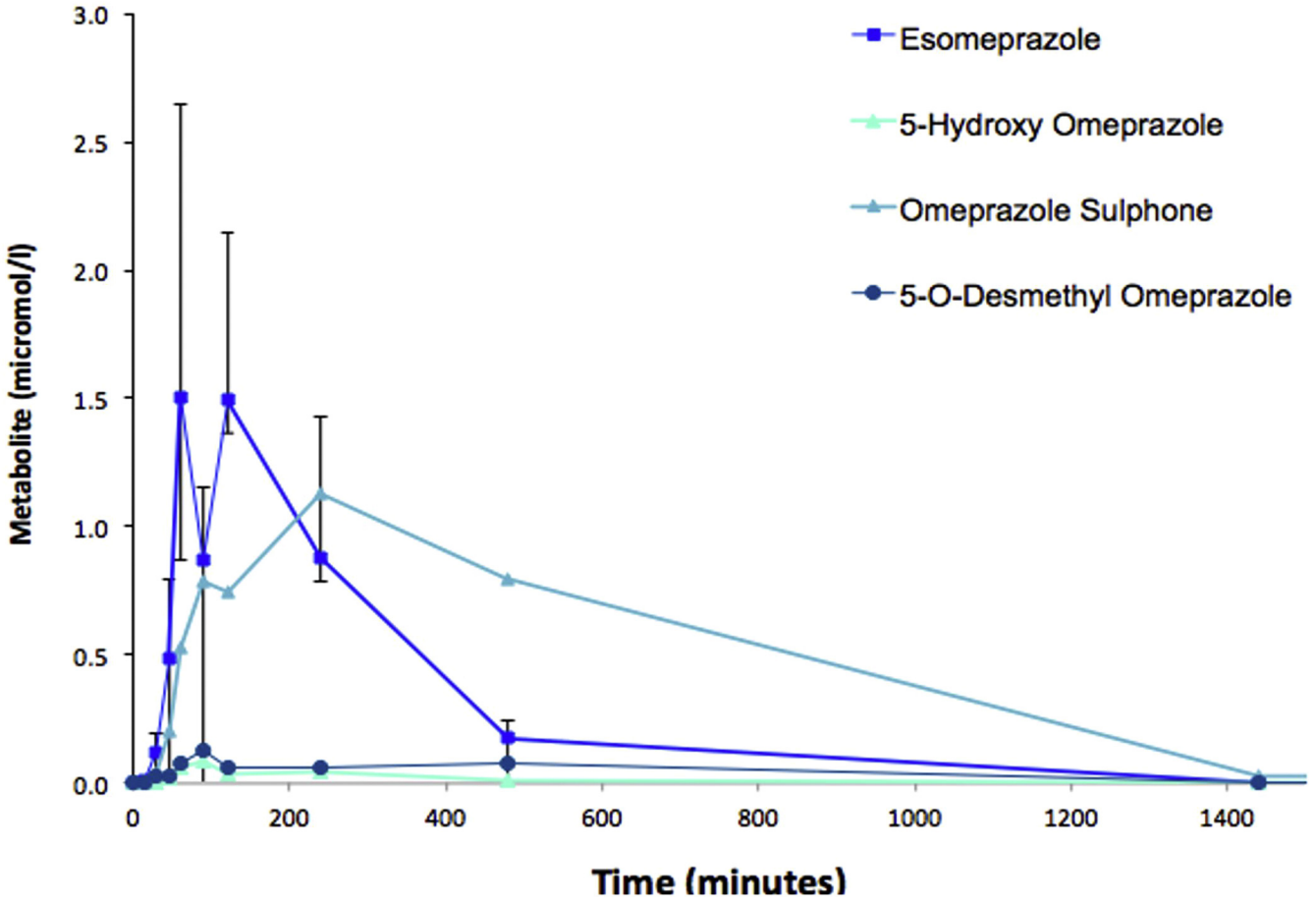
significantly in the circulation of pregnant women with preeclampsia and may have a role in the pathophysiology of the disease. Circulating sFlt1 and soluble endoglin concentrations were extremely high among trial participants, and there were no significant differences in concentrations on serial samples (which were obtained from those who were still undelivered at each time point) between the groups (Figure 3; Figure 4 shows analyte concentrations split into the 2

FIGURE 8
Pharmacokinetic analysis



Pharmacokinetic analysis showed that esomeprazole was detectable in the maternal circulation, with levels peaking soon after administration and a decline in concentration by 500 minutes after administration. Metabolites of esomeprazole (5-hydroxy, 5-O-desmethyl and omeprazole sulphone) were also detectable at lower levels soon after administration with overall higher levels of the metabolite omeprazole sulphone and a steady decrease across the first 1400 minutes.

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gestational age strata). Concentrations of both rapidly declined after delivery, as expected. There were also no differences in circulating levels of PlGF (a proangiogenic factor that is decreased in preeclampsia; Figure 5), endothelin 1 (endogenous vasoconstriction factor that is increased in preeclampsia), or vascular cell adhesion molecule-1 (associated with endothelial dysfunction; Figure 6). Analysis of placental messenger RNA expression of sFlt1, PlGF, vascular endothelial growth factor (proangiogenic factor) and heme oxygenaes-1 (endogenous antioxidant molecule) showed no differences between the esomeprazole and placebo arms (Figure 7).

Esomeprazole pharmacokinetics

Esomeprazole and its metabolites were measured in 10 participants who were assigned randomly to esomeprazole; exposure was similar to that of healthy nonpregnant volunteers with area under the curve geometric means of 5.88 μmol•h/L (95% CI, 2.96−11.68 μmol•h/L; Figure 8).[16] In contrast, esomeprazole and these metabolites were all undetectable in 9 participants who were administered placebo. Concentrations of esomeprazole and the metabolites were extremely low in the umbilical cord blood taken at birth.

Adverse events and adherence

Adherence was excellent. Only 1 participant in the placebo group stopped taking the trial medication. There were no significant differences in the incidences of serious adverse events between the 2 groups (Table 3).

Comment

In our trial, a daily dose of 40 mg of oral esomeprazole did not prolong gestation statistically further than expectant management alone. Additionally, there

TABLE 3
Severe adverse events

Adverse event	Esomeprazole (n=59), n (%)	Placebo (n=60), n (%)	*P* value
Maternal			
Eclampsia	0	3 (5)	.24
Pulmonary edema	1 (1.7)	1 (1.7)	.99
Blood loss of >1000 mL	0	3 (5)	.24
Fetal/neonatal			
Intrauterine death	1 (1.7)	1 (1.7)	.99
Neonatal death	7 (11.9)	9 (15.0)	.67
Necrotizing enterocolitis	4 (6.8)	3 (5)	.68
Neonatal sepsis	9 (15.3)	5 (8.3)	.24
Intracranial hemorrhage	2 (3.4)	0	.15

NOTE: No participant had any of the following serious adverse events: maternal death, severe renal impairment, cerebral vascular event, liver or rupture, posterior reversible encephalopathy syndrome, left ventricular failure, disseminated intravascular coagulation, fetal or neonatal congenital anomaly.

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was no difference in any of the biomarker outcomes or secondary maternal, fetal, and neonatal outcomes, except for placental abruption. However, this is a secondary outcome and did not remain significant on an adjusted analysis.

Ours is only one of very few completed randomized trials to explore treatments for preterm preeclampsia. We have completed perhaps the fastest recruitment for a randomized trial of a drug treatment for preterm preeclampsia, and we achieved this at 1 site by undertaking our study in an area with a very high incidence of disease. It is also the first completed randomized treatment trial of preterm preeclampsia in which blood biomarkers of preeclampsia or endothelial dysfunction were measured, as well as placental messenger RNA expression of genes that are relevant to the pathophysiology of preeclampsia.

There was a nonsignificant trend in median prolongation in the esomeprazole group of 3 days; however, to show that such a difference is significant, we would have needed 402 participants in each arm (alpha error, 5% for 90% power; a post hoc analysis that was calculated from the actual length of gestation observed in the current trial). Despite this, there were no trends in the mean prolongation or the instantaneous hazard of delivery to support this. There was a decrease in the incidence of placental abruption, but this difference was no longer significant after we adjusted for the fact that we performed multiple comparisons for all the different secondary outcomes. Therefore, the significance of this finding, if any, is uncertain.

Esomeprazole is 97% bound to protein and 80% renally excreted. We were concerned that the significant proteinuria that often is associated with preterm preeclampsia may alter esomeprazole pharmacokinetics. Those who received esomeprazole had exposure levels similar to healthy nonpregnant volunteers that had been reported previously.[17] The esomeprazole concentrations that were observed in our participants were around the lower range of concentrations that were used in our preclinical in vitro studies.[6] Thus, although 40 mg may be an optimal dose that is effective in decreasing gastric pH,[18] it is possible that a higher dose or an intravenous dose, which has a higher exposure over time and peak concentration,[16] may be effective in treating preeclampsia.

There is now strong (though circumstantial) evidence that placental secretion of sFlt1 (which causes endothelial dysfunction) may be a significant driver of the disease.[5,19] We and others have pegged decreasing sFlt1 secretion as a strategy to treat preeclampsia.[6,20-24] We did not find changes in any of these markers, which provides biologic evidence to support our clinical findings that 40 mg of oral esomeprazole does not seem to arrest the disease course of preeclampsia once it is diagnosed.

We note that rescuing a pregnancy with advanced preterm disease with severe placental involvement may be a difficult proposition. It has been reported recently that proton inhibitor use, to combat reflux, was associated with decreased sFlt1, soluble endoglin, and endothelin-1 levels.[7] We believe it remains possible that a 40-mg dose may still have merit as a preventative treatment for preeclampsia and may be more realistic. Whether this is the case will also require clinical trials.

Esomeprazole is prescribed widely during pregnancy, and levels in the umbilical cord have not been reported previously. It was reassuring therefore that there was very little, or no, esomeprazole detected in umbilical cord blood that was sampled at birth among those who received the drug. It provides further reassurance that there is likely to be minimal fetal exposure and is consistent with epidemiologic data that show no adverse effects of PPIs on fetal development.[9-11]

There have not been many completed phase II clinical trials that have tested candidate treatments for preterm preeclampsia. Previous trials have met problems with recruitment. One of the main difficulties is that the incidence of disease is low in the developing world. Sildenafil was assessed in a single-site, double-blind randomized controlled trial in Brazil.[25] Over a 28-month period, 100 women were recruited. There was a significant prolongation of gestation in the sildenafil group of 4 days; however, given that sildenafil is a vasodilator, it is possible that this prolongation in gestation may have occurred because the drug decreased blood pressure and mitigated a clinical reason to deliver, rather than temporize disease progression. Antithrombin was assessed to treat preterm preeclampsia in the PRESERVE-1 trial

that enrolled 120 women from 23 tertiary hospitals in the United States over 28 months (ISRCTN23410175).[26] There was no difference in prolongation of pregnancy or composite neonatal outcomes.[27] Trials that have assessed serelaxin (NCT01566630), pravastatin, high doses of antithrombin,[28] and celecoxib (NCT00442676) have been attempted, but all were terminated, perhaps because of poor recruitment.

A potential limitation of our trial is that we were powered to detect a 5-day prolongation of pregnancy and therefore cannot exclude the possibility that 40 mg of esomeprazole may be effective in prolonging pregnancy by 3 days (there was a nonsignificant median difference of 3 days). However, given the findings of pharmacokinetic and biomarker studies, we are inclined to pursue further trials with higher doses rather than to repeat this same trial with a larger number of participants.

Our trial has several strengths. As noted, we performed an integrated trial in which we not only obtained data on clinical outcomes but also derived important insights by undertaking biomarker studies and pharmacokinetics that will inform our next trial. Furthermore, it was run at 1 center, which allowed us to obtain a high recruitment rate, to closely monitor compliance, and to collect uniform high-quality data. Importantly, by basing this trial at an academic center that is embedded within a population with a high incidence of preterm preeclampsia, we overcame the problem faced by previous trials of low recruitment.

In conclusion, in women with a diagnosis of preterm preeclampsia at 26−32 weeks gestation, a daily oral dose of 40 mg of esomeprazole did not prolong pregnancies. Circulating levels of sFlt1 and other antiangiogenic markers were extremely high among the cohort and were not lowered by esomeprazole. The drug appears safe and is well tolerated. In pharmacokinetic studies, we found that esomeprazole was present in the maternal circulation, but concentrations were relatively low compared with those required to elicit tissue/cell responses in our previous laboratory studies. This raises the possibility that higher doses may be effective. Reassuringly, levels of esomeprazole in the umbilical cord blood were very low, or not detectable, which provides further reassurance that very little reaches the fetal compartment.

Furthermore, we have developed and successfully completed a new protocol to evaluate drugs to treat preterm preeclampsia that embeds mechanistic insights and pharmacokinetics with clinical endpoints. We also completed recruitment in a reasonable timeframe by performing this trial in an area where the incidence of preterm preeclampsia is very high. We propose this may be an optimal approach when designing clinical trials for preterm preeclampsia. ■

Acknowledgments

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References

1. Abalos E, Cuesta C, Grosso AL, Chou D, Say L. Global and regional estimates of preeclampsia and eclampsia: a systematic review. Eur J Obstet Gynecol Reprod Biol 2013;170:1–7.
2. Duley LM. The global impact of pre-eclampsia and eclampsia. Semin Perinatol 2009;33:130–7.
3. Baschat AA, Cosmi E, Bilardo CM, et al. Predictors of neonatal outcome in early- onset placental dysfunction. Obstet Gynecol 2007;109:253–61.
4. Levine RJ, Maynard SE, Qian C, et al. Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med 2004;350:672–83.
5. Powe CE, Levine RJ, Karumanchi SA. Preeclampsia, a disease of the maternal endothelium the role of antiangiogenic factors and implications for later cardiovascular disease. Circulation 2011;123:2856–69.
6. Onda K, Tong S, Beard S, et al. Proton pump inhibitors decrease soluble fms-like tyrosine kinase-1 and soluble endoglin secretion, decrease hypertension, and rescue endothelial dysfunction. Hypertension 2017;69:457–68.
7. Saleh L, Samantar R, Garrelds IM, van den Meiracker AH, Visser W, Danser AHJ. Low soluble fms-like tyrosine kinase-1, endoglin, and endothelin-1 levels in women with confirmed or suspected preeclampsia using proton pump inhibitors. Hypertension 2017;70:594–600.
8. Andersson T, Röhss K, Bredberg E, Hassan-Alin M. Pharmacokinetics and pharmacodynamics of esomeprazole, the S-isomer of omeprazole. Aliment Pharmacol Ther 2001;15:1563–9.
9. Matok I, Levy A, Wiznitzer A, Uziel E, Koren G, Gorodischer R. The safety of fetal exposure to proton-pump inhibitors during pregnancy. Dig Dis Sci 2012;57:699–705.
10. Gill SK, O'Brien L, Einarson TR, Koren G. The safety of proton pump inhibitors (PPIs) in pregnancy: a meta-analysis. Am J Gastroenterol 2009;104:1541–5.
11. Pasternak B, Hviid A. Use of proton-pump inhibitors in early pregnancy and the risk of birth defects. N Engl J Med 2010;363:2114–23.
12. Cluver CA, Walker SP, Mol BW, et al. Double blind, randomised, placebo-controlled trial to evaluate the efficacy of esomeprazole to treat early onset pre-eclampsia (PIE Trial): a study protocol. BMJ Open 2015;5:e008211.
13. Hall DR. Understanding expectant management of pre-eclampsia. Obstet Gynaecol Forum 2016;26:22–7.
14. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap): a metadata-driven methodology and workflow process for providing translational research informatics support. J Biomed Inform 2009;42:377–81.
15. Hall DR, Odendaal HJ, Steyn DW, Grove D. Expectant management of early onset, severe pre-eclampsia: maternal outcome. BJOG 2000;107:1252–7.
16. Hassan-Alin M, Andersson T, Bredberg E, Röhss K. Pharmacokinetics of esomeprazole after oral and intravenous administration of single and repeated doses to healthy subjects. Eur J Clin Pharmacol 2000;56:665–70.
17. Chunduri RHB, Dannana GS. Development and validation of a high throughput UPLC−MS/MS method for simultaneous quantification of esomeprazole, rabeprazole and levosulpiride in human plasma. J Pharm Anal 2016;6:190–8.
18. Röhss K, Hasselgren G, Hedenström H. Effect of esomeprazole 40 mg vs omeprazole 40 mg on 24-hour intragastric ph in patients with symptoms of gastroesophageal reflux disease. Dig Dis Sci 2002;47:954–8.
19. Maynard SE, Min JY, Merchan J, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest 2003;111:649–58.
20. Brownfoot FC, Tong S, Hannan NJ, Hastie R, Cannon P, Kaitu'u-Lino TJ. Effects of simvastatin, rosuvastatin and pravastatin on soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sENG) secretion from human umbilical vein endothelial cells, primary trophoblast cells and placenta. BMC Pregnancy Childbirth 2016;16:117.

21. Brownfoot FC, Hastie R, Hannan NJ, et al. Metformin as a prevention and treatment for preeclampsia: effects on soluble fms-like tyrosine kinase 1 and soluble endoglin secretion and endothelial dysfunction. Am J Obstet Gynecol 2016;214:356.e1–15.

22. Brownfoot FC, Tong S, Hannan NJ, et al. Effects of pravastatin on human placenta, endothelium, and women with severe preeclampsia. Hypertension 2015;66:687–97;discussion 445.

23. Hannan NJ, Brownfoot FC, Cannon P, et al. Resveratrol inhibits release of soluble fms-like tyrosine kinase (sFlt-1) and soluble endoglin and improves vascular dysfunction - implications as a preeclampsia treatment. Sci Rep 2017;7:1819.

24. Thadhani R, Kisner T, Hagmann H, et al. Pilot study of extracorporeal removal of soluble fms-like tyrosine kinase 1 in preeclampsia. Circulation 2011;124:940–50.

25. Vigil-De Gracia P, Ludmir J. Perinatal and hemodynamic evaluation of sildenafil citrate for preeclampsia treatment. Obstet Gynecol 2016;128:1181–2.

26. Paidas MJ, Sibai BM, Triche EW, Frieling J, Lowry S; PRESERVE-1 Study Group. Exploring the role of antithrombin replacement for the treatment of preeclampsia: a prospective randomized evaluation of the safety and efficacy of recombinant antithrombin in very preterm preeclampsia (PRESERVE-1). Am J Reprod Immunol 2013;69:539–44.

27. Sibai B, Paidas MJ. LB02: randomized double-blind placebo controlled evaluation of the safety and efficacy of recombinant antithrombin vs placebo in preterm preeclampsia. Am J Obstet Gynecol 2017;216(suppl):S559–60.

28. D'Angelo A, Valsecchi L. High dose antithrombin supplementation in early preeclampsia: a randomized, double blind, placebo-controlled study. Thromb Res 2016;140:7–13.

Author and article information

From the Department of Obstetrics and Gynaecology, Stellenbosch University and Tygerberg Hospital, (Drs Cluver, Theron, and Hall, and Ms van Papendorp); the Division of Clinical Pharmacology, Department of Medicine, Stellenbosch University and Tygerberg Hospital (Dr Decloedt); the Division of Chemical Pathology, Stellenbosch University and National Health Laboratory Service (Dr Rensburg and Mr Schubert), Cape Town, South Africa; the Translational Obstetrics Group (Drs Cluver, Hannan, Walker, and Tong and Ms Beard), Mercy Perinatal (Drs Hannan, Walker, and Tong and Ms Beard), and the Department of Anaesthetics (Dr Hiscock), University of Melbourne, Mercy Hospital for Women, Melbourne, Victoria, Australia; the Department of Obstetrics and Gynaecology, Monash School of Medicine, Monash University, Melbourne, Victoria, Australia (Dr Mol); LCMS Central Analytical Facility and Department of Biochemistry, Stellenbosch University, Stellenbosch, South Africa (Dr Stander and Ms Adams).

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Corresponding author: Catherine Anne Cluver, MMed. cathycluver@hotmail.com

Supplemental Material

Measuring plasma concentrations of esomeprazole and its metabolites

Plasma concentrations of esomeprazole and its metabolites (5-hydroxy omeprazole, omeprazole sulphone, and 5-O-desmethyl omeprazole) were determined with the use of a validated ultra-performance liquid chromatography–tandem mass spectrometry method. A Waters Acquity ultra-performance liquid chromatograph (Waters Corporation, Milford, MA) with a Waters HSS T3 column was linked to a Xevo TQ-S mass spectrometer (Waters Corporation). A gradient of 0.1% formic acid to acetonitrile was used, with d3-esomeprazole as the internal standard. In brief, the drugs were extracted with a buffered (2 mmol/L ammonium formate; pH 5.5) acetonitrile 60% solution, and the precipitated plasma proteins were separated by centrifugation (12 000*g*). The intra- and interday accuracy of the quality control samples was >90% and 85%, respectively; the intra- and interday precision was <11% and <15%, except for 5-O-desmethyl omeprazole that was 20% at the lower limit of quantification. The limit of quantitation was 1 ng/mL for all analytes. Phoenix WinNonlin software (version 9.0; Certara, Princeton, NJ) was used to characterize the pharmacokinetic parameters of esomeprazole with the use of non-compartmental analyses. The area under the plasma concentration-time curve was calculated for the 24-hr dosing interval with the log-linear trapezoidal method. Pharmacokinetic data were summarized as geometric mean values with 95% confidence intervals.

Preparation of placental tissue for analysis

Placental tissue was dissected from the whole placenta. Four pieces were dissected from distant sites; the tissue pieces were then washed in sterile phosphate-buffered saline solution, and smaller pieces were then dissected (to allow appropriate penetration of RNA preservation buffer [RNAlater]). Each piece was immersed in RNAlater according to manufacturer's instruction. Tissue samples were then blotted dry, snap frozen, and stored at −80°C until subsequent analysis.

Measuring analytes in the plasma by enzyme-linked immunosorbent assay

Patient plasma was assessed with the use of enzyme-linked immunosorbent assay for the presence of the following soluble factors: soluble Flt-1 (DuoSet VEGF R1/Flt-1 kit; R&D Systems by Bioscience, Waterloo, Australia), soluble endoglin (DuoSet Human Endoglin CD/105; R&D Systems), placental growth factor (P DuoSet PlGF; R&D Systems), endothelin-1 (Quantikine endothelin-1; R&D Systems), and soluble vascular cell adhesion molecule-1 (human VCAM-1/CD106 DuoSet; R&D Systems). Optical density for enzyme-linked immunosorbent assays was determined with a BioRad X-Mark microplate spectrophotometer (BioRad Laboratories, Inc, Hercules, CA). Protein levels were determined with BioRad Microplate Manager software (version 6; BioRad Laboratories, Inc).

Measuring expression of genes in placental tissue

Total RNA was extracted from placental tissue (from placebo [n=32] and esomeprazole [n=33] treated women) with the use of the RNeasy mini kit (Qiagen, Valencia, CA) and was quantified with a Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE). RNA (0.2 μg) was converted to complementary DNA with the use of a high-capacity complementary DNA reverse transcriptase kit (Applied Biosystems Life Technologies Corporation, Carlsbad, CA), according to manufacturer guidelines.

Quantitative polymerase chain reaction was performed with the use of Taqman gene expression assays for the following genes: *sFlt1*, *HO-1*, *PlGF* and *VEGFA*. Polymerase chain reaction was performed on the CFX 384 (BioRad Laboratories, Inc) using FAM-labeled Taqman universal polymerase chain reaction mastermix (Applied Biosystems) with the following run conditions: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, 60°C for 1 minute (40 cycles). All data were normalized to the housekeeping genes *TOP1* and *CYC1* as an internal control and calibrated against the average cycle threshold of the control samples. The results were expressed as fold-change relative to control subjects. All samples were run in triplicate.

Addendum C: Study protocol

RESEARCH PROTOCOL

Ethics Reference #: S17/05/107

Project title:	PHARMACOKINETIC STUDY OF ACETYL-PAS AND GLYCINE-PAS AND THE CORRELATION BETWEEN THE RATE OF METABOLISM AND THE DEVELOPMENT OF TOXICITY IN MDR- and XDR-TB PATIENTS TREATED WITH PAS
Investigator:	Kim Terry-Ann Adams for MSc project
Place of research:	Division of Clinical Pharmacology, Department of Medicine, Faculty of Health Sciences, Stellenbosch University
Supervisor:	Prof H Reuter, Division of Clinical Pharmacology, Department of Medicine, Faculty of Medicine and Health Sciences, Stellenbosch University
Co-supervisor:	Dr M Stander, Central Analytical Facility, Stellenbosch University
Collaborators:	Prof P Donald, Division of Paediatrics and Child Health, Department of Medicine, Faculty of Medicine and Health Sciences, Stellenbosch University

1. PROTOCOL SYNOPSIS

1.1. Title

Pharmacokinetic study of metabolites acetyl-PAS and glycine-PAS and the correlation between the rate of metabolism and the development of toxicity in MDR and XDR-TB patients treated with PAS.

1.2. Introduction

Para-aminosalicylic acid (PAS), is a bacteriostatic chemotherapeutic agent used in the therapy of all forms of tuberculosis (TB). It is readily absorbed in the gastrointestinal (GI) tract and primarily metabolised in the liver to, N-acetyl-para-aminosalicylic acid (APAS) and p-aminosalicyluric acid (GPAS). Since its clinical introduction in the late 1940's, PAS was part of the standard first-line TB treatment for over 20 years. Along with isoniazid (INH) and streptomycin, PAS showed substantial efficacy and prevention of drug resistance in companion drugs. However, it was notorious for gastrointestinal intolerance, causing nausea, vomiting and abdominal discomfort. Hereby, PAS was replaced with other efficacious drugs that showed better tolerance.

Fuelled by the HIV pandemic, increasing rates of multidrug-resistance (MDR) and also extensively drug-resistance (XDR) to first-line TB agents, the use of available drugs needs to be revised. Because of the relative lack of use of PAS over the past three decades, most strains of *Mycobacterium tuberculosis* remain susceptible to it. This has urged the resurgence of PAS in the management of patients with MDR-TB.

When PAS was introduced in anti-TB management, it was administered in divided daily doses. Several groups later turned to once daily dosing and found favourable results. Lehmann, later also advocated a single daily dose, emphasising the greater PAS exposure brought about allowed greater drug penetration into tuberculosis tissue (Lehmann 1969). The results of the single study of PAS in a once daily 15 g dose suggest that, although usually thought bacteriostatic, PAS might have bactericidal activity if high enough concentrations are reached (Jindani *et al.* 1980).

In a study by Sy *et al.* (2015), the safety and tolerability of two dosing regimens (8 g once-daily vs 4 g twice-daily) was investigated. No distinctive difference in the extent of the side effects

could be found between the two regimens. The results indicated that PAS adverse gastrointestinal (GI) effects were not necessarily attributed to higher PAS concentrations seen with the once-daily regimen. It was rather found that once-daily dosing was associated with less intolerance versus the twice daily dosing. These findings are in agreement with earlier studies by the British Medical Council (1952). Sy *et al*. did however observe the influence of other characteristics of pharmacokinetics such as C_{min} (minimum drug plasma concentration), which correlated with abdominal pain, discomfort and diarrhoea.

These findings have led to the speculation that factors such as absorption and metabolism may well be related to the GI effects observed in treatment with PAS. The current study therefore hypothesises, that more rapid absorption of PAS and its biotransformation to APAS may be the causative of increased GI adverse effects, as opposed to the notion of high serum PAS concentrations.

1.3. Aims and Objectives

The current study aims to establish a practical method for the simultaneous clinical monitoring of PAS and its metabolites APAS and GPAS in human plasma.

⇒ By developing and validating an analytical method using ultra-performance liquid chromatography (UPLC) in tandem with triple quadrupole mass spectrometer (MS).

It aims to study the *in vivo* pharmacokinetics (PK) of metabolites APAS and GPAS in MDR and XDR TB adult patients.

⇒ By measuring the levels of APAS and GPAS in plasma, using the developed LC-MS/MS method.

It aims to evaluate the possible cause of adverse reactions experienced in patients treated with PAS.

⇒ By determining if a correlation exists between high or low levels of APAS and GPAS and reported events of gastrointestinal intolerance (nausea, bloating, diarrhoea, abdominal pain). This data was previously collected (M12/01/006). The events were rated using a 10-point visual analogue scale (VAS).

1.4. Study Design

As part of an ongoing study (HREC #: M12/01/006), the same PAS samples will be analysed for determination of its metabolites. Therefore, this two-period, cross over study will be conducted in a population with high prevalence of drug resistant TB and on a TB regimen which includes PAS. Forty one (41) adults at Brooklyn Hospital for Chest and Diseases, Cape Town, will be enrolled in the study. They will be divided into two groups with the following PAS dosing regimen: 4 g twice-daily and 8 g once-daily, for a period of 8 days (Days 1-8). Thereafter, the regimens will be crossed over and followed for another 8 days (Days 9-16).

Blood samples will be collected over a 24 hour period on Day 8 and Day 16, and dosing regimen, dosing time, blood sampling times and demographic characterises will be recorded for each patient. Three millilitre (3 mL) blood specimens will be collected at time 0, 1, 2, 3, 4, 6, 8, 12 (pre-dose) and 24 hours after dosing.

1.5. Sample Stability

The stability of the samples (stored at - 80˚C) will be evaluated by comparing the PAS levels achieved previously by de Kock *et al.* (2013) to those quantified in the current study. Internal standard drug for PAS, APAS and GPAS will be included in the analytical analysis as control standards.

1.6. Methodology

A method will be established and validated according to bio-analytical method validation guidance by U.S Food and Drug Administration (FDA), under the supervision of Dr Marietjie Stander (Director of LCMS unit, Central Analytical Facilities, Stellenbosch University).

The samples will be analysed for APAS, GPAS and parent PAS, at t0, 1, 2, 3, 4, 6, 8, 12 (pre-dose) and 24 hours post dose. The MS/MS assay will be determined using a Waters Xevo TQ triple quadrupole mass spectrometer (MS) connected to a Waters Acquity Ultra performance liquid chromatograph (UPLC) system equipped with an appropriate analytical column.

1.7. Ethical Considerations

The data and information of each patient will remain concealed by use of a unique subject code, previously allocated. The patient confidentiality will be maintained at all times allowing only researcher and authorized official's access to personal information.

No additional consent is required from the patients as no specific limit on the period of analysis of the stored specimen was disclosed.

2. LITERATURE REVIEW

Tuberculosis is a chronic infectious disease cause by the acid-fast bacillus *Mycobacterium tuberculosis* (Kvasnovsky *et al.* 2011). According to the 2015 global TB report the epidemic accounts for 10.4 million cases and 1.4 million deaths worldwide (*Global Tuberculosis Report:* WHO report 2015). The World Health Organization (WHO) has classified TB endemic in the developing world, with 80% of all cases occurring in high burdened countries, including South Africa.

Multidrug-resistance tuberculosis (MDR-TB) caused by strains of *M. tuberculosis* resistant to first-line TB drugs, rifampicin and isoniazid (INH), has emerged as an increasing threat in TB management (Wells *et al.* 2007, Iseman 2002). Additionally, extensively drug-resistant tuberculosis (XDR-TB) drugs, are required in isolates resistant to second-line drugs such as fluoroquinolones and injectable drugs kanamycin and capreomycin (Kvasnovsky *et al.* 2011).

Fuelled by the HIV pandemic and increasing rates of resistance to established anti-TB drugs, several new drugs have now completed clinical trials in patients with drug-resistant TB. However, even if these drugs are successful in shortening treatment duration, the need for protection from the emergence of resistance needs to be maintained. Due to these circumstances, the use of available drugs needs to be revised. This has urged the re-introduction of para-aminosalicylic acid (PAS), one of the earliest efficacious anti-TB chemotherapeutic agents used.

2.1. PAS

Since its clinical introduction in the late 1940's, PAS has formed part of the standard treatment of TB for decades. PAS is classified as a bacteriostatic agent and has shown to inhibit growth of *M. tuberculosis* at a concentration of 1-2 µg/mL^2, in vitro (Peloquin *et al*. 1994, Annabel 2017). In a study conducted in healthy individuals, a mean maximum concentration (C_{max}) of 20 µg/mL with a mean peak time of 6 hours was achieved after a single 4 g PAS dose, in a granular slow release formulation (Lehmann 1969, PASER 1996, Cynamon 2001).

2.2. Pharmacokinetics of PAS

PAS is readily and completely absorbed from the gastrointestinal (GI) tract, 50-73% bound to plasma proteins and well distributed systemically (Momekov *et al*. 2015). It is then metabolised beginning in the gut wall and primarily in the liver. The main pathway of PAS metabolism comprises conjugating processes (Figure 1), of three different active groups in the benzene ring. The two major metabolites are formed by conjugation of the amino group (-NH_2) in position 4 and the carboxyl group (-COOH) in position 1 (Lehmann 1969, Momekov *et al.* 2015).

Following an oral dose, PAS has a half-life of approximately 1 hour. Concentrations in the plasma are negligible within 4-5 hours post dose (Berning and Peloquin 1998). Within 24 hours, more than 80% of the dose is excreted in the urine. Apart from glomerular filtration the half of the dose and its metabolites are eliminated by tubular secretion (Otten *et al.* 1975).

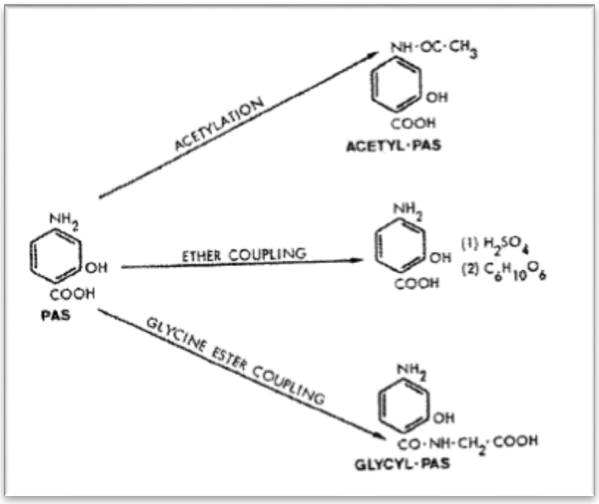

Figure 1: Schematic of the alternative conjugations of PAS. Adapted from Lehmann 1969.

2.3. Biotransformation of PAS

The 3 functional groups in the benzene ring of PAS are crucial for the anti-tuberculosis activity of PAS (Lehmann 1969). The conjugation of the two main metabolites occurs as follows:

⇒ N-Acetylation

The amino group (-NH2) at position 4 is conjugated with acetyl Co-enzyme A (Co-A) to form acetyl-PAS (APAS). APAS is the primary metabolite of PAS, and is known to be devoid of bacteriostatic activity against *M. tuberculosis* (Momekov *et al.* 2015). The rate of this reaction is modulated by N-acetyltransferase 1 (NAT1) which is encoded by the gene *NAT1* (Figure 2).

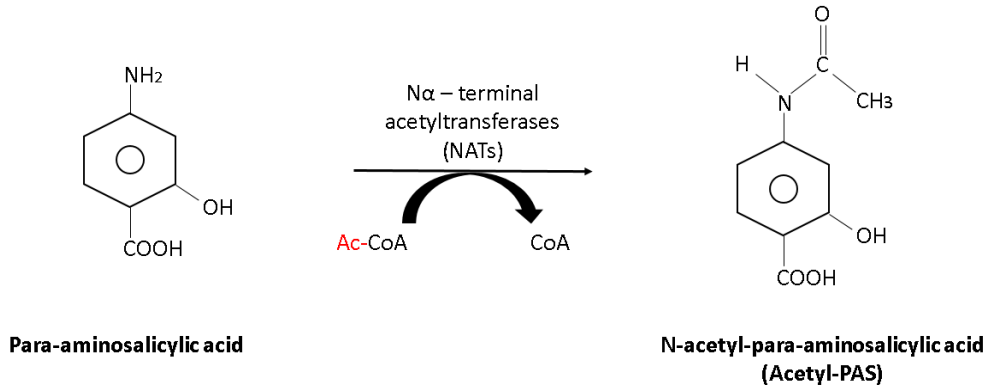

Figure 2: Schematic adapted from Wang *et al*. 2016.

Isoniazid (INH) metabolism is catalysed by N-acetyltransferase 2 (NAT2), the isoenzyme of NAT1 (Butcher *et al.* 2002). Shortly after introduction of INH in anti-tuberculosis treatment,, substantial differences in its N-acetylation activity was found to be due to a polymorphic NAT2 (Sim *et al*. 2008).

The biotransformation of PAS was thought to be unimodal, but after the sequencing of NAT1, several polymorphisms affecting NAT1 activity were identified thus PAS N-acetylation is considered polymorphic like that of INH (Blum *et al*. 1990).

⇒ Glycine-conjugation

Glycine N-acyltransferase (GLYAT), is the enzyme responsible for glycine conjugation of several xenobiotic organic acids (Badenhorst *et al.* 2013). However, due to a small number of pharmaceutical drugs conjugated to glycine, the pathway has not yet been

characterized in detail. The following pathway was adapted from that of salicylic acid (aspirin). First PAS is ligated to Co-A to form a high energy salicyl-CoA thioester. Thereafter, salicyl-CoA is conjugated to a glycine amino acid by GLYAT to form aminosalicyluric acid, and releasing Co-A (Figure 3) (Knights et al. 2007). Glycine-PAS has shown anti-tuberculosis activity that varies between 50% and 75% that of PAS, depending on the dosage of PAS (Lehmann 1969). Single nucleotide polymorphisms (SNPs) have been observed in human GLYAT, but the influence on the enzyme activity is not clear.

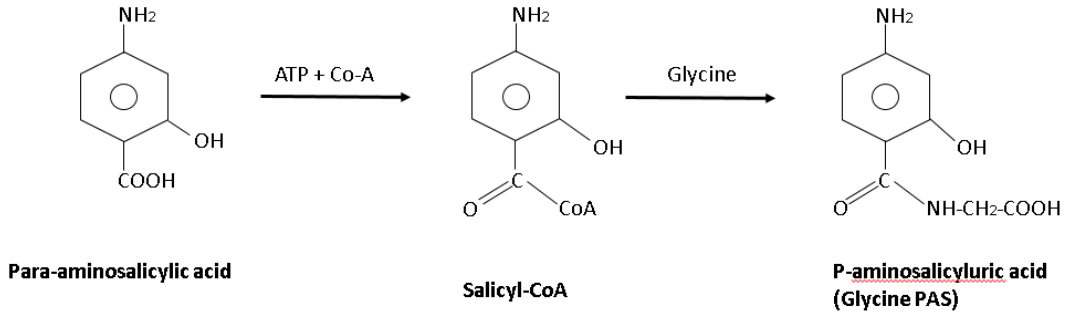

Figure 3: Schematic adapted from Badenhorst *et al*. 2013.

In studies by Lipman (1945) and Chantrenne (1951), acetylation was least when glycine conjugation is at its maximum. Lehmann (1969), observed the same shift in processes. When glycine conjugation declines, acetylation continues long after. This phenomenon was explained by the competition for a common substrate, presumably CoA. At a low dose, PAS absorption is accompanied by first-pass rapid acetylation beginning in the gut wall, therefore APAS may be detected in the blood before PAS itself (Peloquin et al. 1994). However, at high doses acetylation is saturated due to scarcity of available Co-A, inhibiting the enzyme function. This allows PAS and GPAS concentrations to rise expressively higher while APAS is relatively low (Dubovsky and Lehmann 1991, Lehmann 1946). In the same way, INH acetylation will be repressed during PAS therapy, allowing its concentrations to be higher when INH and PAS are administered together (Lauener and Favez 1959, Johnson and Corte 1956, Morse et al. 1956). These findings have emphasis the substantial effect of PAS dosage.

2.4. PAS tolerance and safety

Shortly after the introduction of PAS, intolerance was noted with common adverse effects manifested in nausea, vomiting, diarrhoea, anorexia, abdominal pain and cramping (Lehmann 1949). During the MRC studies done on PAS, it was evident that PAS dose affected intolerance. 12%, 15%, and 52% of patients receiving 5 g, 10 g and 20 g daily, reported GI intolerance respectively (MRC 1952). In another investigation by Marsden (1954), 119 (30%) of 399 patients reported GI intolerance when administered divided PAS doses, but only in 44 (12%) of 383 patients when given a once-daily dose. Similar findings were reported by Riska from 1954-1962, as better or equivalent tolerance with PAS given once-daily compared with the same dose in divided doses.

In an attempt to improve intolerance, a granular slow release PAS formulation (GSR-PAS) was introduced in the USA, and made available for treatment of drug resistant TB, in South Africa (Donald and Schaaf 2007). GSR-PAS seemed to be better tolerated with fewer patients reporting moderate nausea, vomiting, bloating and diarrhoea (Peloquin 1994, 1999). A similar investigation by Sy *et al.* (2015), compared GSR-PAS 4 g twice-daily vs 8 g once-daily. Little difference in tolerance was observed, a result similar to other studies. However, the influence of other pharmacokinetic characteristics such as the C_{min} (minimum observed plasma concentration) of PAS, correlated with abdominal pain, discomfort and diarrhoea.

2.5. PAS optimal dosing regimen

When PAS was first introduced in anti-TB management, it was administered in divided daily doses. Several groups later turned to once daily dosing, a change supported by Lehmann (1969) who stated that the greater PAS exposure brought about the greater the drug penetration into tuberculosis tissue. In a study by Jindani *et al.* 1980 which investigated the early bactericidal activity of PAS in a once daily 15 g dose, reported that although PAS is thought to be bacteriostatic, it may have bactericidal activity if high enough concentrations are reached. The prevention of resistance in companion drugs is often related to bactericidal activity. This may explain the improved activity of a higher individual dose of PAS for preventing resistance shown in earlier trials by the British MRC in the absence of INH (MRC 1952, Daniels and Hill 1952).

3. RATIONALE OF STUDY

As part of an ongoing study (#M12/01/006) to report the pharmacokinetics of PAS which lacks available data, the current study aims to determine the pharmacokinetics of the major metabolites of PAS. Due to the potential bactericidal characteristics of PAS at a higher dose regimen, there is an urgent need to shed light on the factors that contribute to gastrointestinal intolerance associated with PAS treatment. This study aims to suggest a regimen that uses PAS dosing optimally for better management of DR-TB cases co-infected with HIV. If we were able to elicit the correlation between the serum concentration of PAS metabolites and GI effects, it could add to our understanding of the individual pharmacokinetic profile of PAS which may be greatly affected by the enzymes responsible for the rate of its biotransformation.

4. AIMS

⇒ The main aim of this research project is to study the *in vivo* pharmacokinetics (PK) of metabolites N-acetyl-para-aminosalicylic acid (acetyl-PAS, APAS) and p-aminosalicyluric acid (glycine-PAS, GPAS) in adults. The study aims to establish whether high plasma concentrations of these metabolites are associated with gastrointestinal intolerance?

⇒ The second aim of this study for a MSc degree is to develop a sensitive and high throughput LC-MS/MS method for the simultaneous quantification of para-aminosalicylic acid (PAS) and its major metabolites (APAS) and (GPAS), in human plasma. The analytical method will be developed on an ultra-performance liquid chromatography system (UPLC) in tandem with triple quadrupole mass spectrometer (MS). Such a system is a proven analytical tool that offers very low limits of quantification, together with high throughput and excellent specificity.

5. OBJECTIVES

⇒ The primary objective is to measure the levels of APAS and GPAS in plasma, using the developed and validated LC-MS/MS analytical method. This assay will indicate the pharmacokinetic profile of subjects under two dosing regimens (8 g once-daily vs 4 g twice-daily).

⇒ The secondary objective is to determine if high or low levels of APAS and GPAS contribute to toxicity. This will be done by comparing the measured levels to a 10-point visual analogue scale (VAS) which rates adverse effects including abdominal pain, nausea, diarrhoea and bloating.

6. STUDY METHODOLOGY

As part on an ongoing study (HREC #: M12/01/006) by de Kock *et al*. (2013), a selection of the same PAS samples will be analysed to assay the major metabolites of the parent drug. Therefore, the current study is a randomized two period cross over study which will report the pharmacokinetics of metabolites APAS and GPAS under two dosing regimens: 4 g twice-daily and 8 g once-daily in adult patients. The study will be conducted in a population based on inclusion criteria of high prevalence of drug resistant (DR) TB, HIV positive or negative status and a treatment regimen which includes PAS.

6.1. Sample size

Adult patients, from the Brooklyn Hospital for Chest and Diseases in Cape Town, who are routinely screened for DR-TB and treated with PAS, will be enrolled in this study. All patients in the larger study will be included (41) minus 11 patients due to the loss of specimen during the previous study analysis.

6.2. Sample collection

The blood samples will be collected for pharmacokinetic analysis over a 24 hour period on Day 8, after which the regimens will be crossed over. The patients who received 2x 4 g GSR-PAS will then receive 1x 8g GSR-PAS (PASER® granules) and vice versa. These regimens will be

followed for another 8 days (Day 9-16). The blood samples will be collected on Day 16. Three millilitres (3mL) of blood specimen will be collected through a catheter inserted into a forearm vein over 24 hours as follows: t0, 1, 2, 3, 4, 6, 8, 12 (pre-dose) and 24 hours after post dose.

6.3. Sample criteria

- Male and female patients aged ≥ 18 years
- Race and gender data will be collected
- Known HIV status has to be advised or obtained before enrolment
- It is required of the patient to be on a clinically stable and unchanged regimen which includes PAS as part of their treatment for at least 14 days
- The following data will be collected at enrolment: TB disease status, disease spectrum, mycobacterial culture and drug susceptibility test pattern, concurrent illness, concomitant medication, age, Body Mass Index (BMI), blood pressure, liver and renal function
- And physical examination will take place before commencement of the study

6.4. Informed Consent

De Kock *et al.* set up two consent forms for research involving genetic analysis (DNA extraction from plasma sample) and pharmacokinetic analysis using the same sample. In the form for genetic studies the storage time is worded ambiguously, but as said in the last sentence, the sample will be stored for a minimum of 2 years. We acknowledge that this was not written very well and apologise sincerely. In terms of the pharmacokinetic analysis, de Kock did not specify a limit on the period that the sample may be stored or analysed. This is due to the fact that there was a lack of funding and LC-MS equipment available at the time for more extensive PK analysis.

Therefore, with the support of NRF funding from Prof Peter Donald and a new LC-MS system at Clinical Pharmacology we can now look at not only the parent PAS but also its metabolites, as we would have hoped to do with the initial study. No additional consent is thus required from the patients as we are doing the exact same pharmacokinetic analysis of the samples, using more advanced analytical procedures. No DNA or protein analysis will be performed on these samples.

How long will your blood be stored and where will it be stored?

The sample will be stored for 2 years depending on the results of the study.
Your specimen will be stored at the University of Stellenbosch Medical School in a dedicated fridge or freezer for at least 2 years or at the laboratory that performed the same test(s) as a routine service.

If your blood is to be stored is there a chance that it will be used for other research?

Your specimen will only be used for the genetic research as indicated above and any further testing will only be done after obtaining full written consent. Also, if the researchers wish to use your stored specimen for **additional research in this field** they will be required to apply for permission to do so from the Human Research Ethics Committee at Stellenbosch University (that can be contacted at telephone number 021 938 9657).

7. SAMPLE STABILITY

The stability of the samples (stored at - 80˚C) will be determined by comparing the PAS levels achieved previously by de Kock *et al.* (2013) to those quantified in the current study. Internal standard drug for PAS and acetyl-PAS will be purchased while glycine-PAS will be synthesised by Laboratory of Organic Chemistry (Stellenbosch University) and used in the LC-MS/MS analysis as a control standard. These control standards will also be tested for purity prior to analysis. Stability will also be assessed by dual analysis at Central Analytical Facilities (CAF, Stellenbosch University) and the Clinical Pharmacology Bioanalytical Laboratory (Tygerberg Medical Campus, Stellenbosch University).

8. METHOD DEVELOPMENT

The purpose of this section of the study is to develop a sensitive and high throughput LC-MS/MS method for the simultaneous quantification of para-aminosalicylic acid (PAS) and its major metabolites (APAS) and (GPAS), in human plasma. The analytical method will be developed on an ultra-performance liquid chromatography system (UPLC) in tandem with triple quadrupole mass spectrometer (MS). Such a system is a proven analytical tool that offers very low limits of quantification, together with high throughput and excellent specificity.

With this type of system, small sample volumes can be used and relatively shorter run times established therefore it offers a practical method for the clinical monitoring of the therapeutic agent PAS and its metabolites in TB patients.
For the successful development of a method, the following prerequisites will be met, selection of a qualified and calibrated instrument, purchasing of reliable reference standards and the selection and integrity of the sample. The common steps that we will follow in the method development are as follows:

1) Standard analyte characterization
2) Method requirements
3) Literature search
4) Selecting the method based on a suitable solvent
5) Instrumental (LC and MS) setup and preliminary studies
6) Optimization of parameters on the instrument
7) Documentation of analytical figure which may include chromatographs
8) Evaluation of the method development with the sample specimen (plasma)
9) Determination of percent recovery of the sample using analytical performance characteristics including specificity, accuracy, linearity, limit of detection (LOD), quantification limits (LOQ), range, and precision.
10) Demonstration of quantitative sample analysis

The method performance will be assessed according to the Guidance for Industry Bioanalytical Method Validation criteria (published by Food and Drug Administration – FDA). The method will also be governed for quality standards by Good Laboratory Practice (GLP) regulations.

9. PHARMACOKINETIC ANALYSIS

A pharmacokinetic profile for APAS, GPAS and PAS will be generated under each dose regimen in order to visually determine any differences between the two occasions. Assays at sample collection times t0, 1, 2, 3, 4, 6, 8, 12 and 24 taken on Day 8 and Day 16 will be used to determine the following pharmacokinetic parameters.

- ⇒ Area under the concentration-time curve (AUC_{0-24}) post dose on Day 8 and Day 16
- ⇒ Maximum observed concentration (C_{max}) from time 0 to 12 hours post dose
- ⇒ Time to reach the maximum observed concentration (t_{max})
- ⇒ Minimum observed concentration (C_{min}) from time 0 to 12 hours post dose

10. STATISTICAL ANALYSIS

10.1. Sample size

The sample size remains the same as the initial study, minus 11 patients due to specimen loss. Sample size therefore requires no power analysis as only 30 patients are available for the proposed study.

10.2. Data management

The data will be captured using Microsoft excel 2016, which applies to all demographic data (gender, age, race, BMI, height, weight, concomitant medication, HIV status, and treatment regimen). A code will be allocated to all grouped data.

Security access will be maintained on this data, using an access code. The data and information of each patient will remain concealed by use of a unique subject code, previously allocated. The patient confidentiality will be maintained at all times allowing only researcher and authorized official's access to personal information. All the collected data will be backed up on external drives and iCloud.

10.3. Data analysis

Microsoft excel will be used to capture demographic data (age, weight, height, BMI, concomitant medication), categorical data (gender, race, HIV status, concurrent illness). Descriptive statistics will be used to summarise the data, using STATA 14 (data analysis software system. Demographical data will be measured for normality and described by mean and standard deviation (SD) in the form of histograms or scatter plots. If the demographics are not normally skewed, it will be described by medians and IQR (25^{th} and 75^{th} percentile) using line graphs. Categorical data will be measured by quality data tests and presented in frequencies using bar graphs.

The pharmacokinetic profile for APAS and GPAS generated from t0, 1, 2, 3, 4, 6, 8, 12 and 24 hours will be used to determine AUC (area under the concentration-time curve), Cmax (maximum concentration), and Cmin (minimum concentration). The plasma concentrations will be captured in Microsoft excel and summarised by descriptive statistics (including number of subjects, mean, SD, percent co-efficient of variation (CV %), 25^{th} and 75^{th} median percentile, minimum and maximum), and the median on each time point will be calculated using STATA 14.

The safety and tolerability data, generated by a 10-point Visual Analogue Scale (VAS) will be summarised by descriptive statistics. The symptoms (abdominal pain, nausea, bloating and diarrhoea) will be summarised as a grouped under yes/no to determine GI effects. Therefore, if any of the symptoms were experienced that classifies as an adverse effect.

11. TIME PLAN

2017	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct
	Register	Protocol write-up			Ethical Approval	Start study	Method development and validation		
2018	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
	PK and Data analysis			Write-up				Prepare for submission	

12. STRENGTHS AND LIMITATIONS

With use of an LC-MS system, a smaller plasma volume is required which allows for the possibility of repeat analysis when validating the results of the analytical method

The current study sample size has been reduced from the initial study conducted in 2013 due to loss of sample during analysis procedures.

13. ETHICAL CONSIDERATIONS

The project will be conducted accordingly to South African laws and regulations, including South African GCP and to the Declaration of Helsinki.

The approval notice for the main study conducted by De Kock et al 2013 (M12/01/006) has been attached to the protocol. The data and information of each patient will remain concealed by use of a unique subject code, previously allocated. The patient confidentiality will be maintained at all times allowing only researcher and authorized official's access to personal information.

14. FUNDING AND BUDGET

Partial funding from the departmental funds have been made available. In addition, funding will be requested from grants provided to Prof PR Donald. Funding requests/applications will be submitted to the Wellcome Trust. A proposed budget has been set up as follows:

DESCRIPTION	AMOUNT
Consumables:	
Reagents	
Laboratory analysis:	
Development and validation	
LC-MS specimen analysis	
Glycine-PAS synthesis	
Courier service for samples	
TOTAL	

15. REFERENCES

1. Kvasnovsky C, Cegielski J, Erasmus R, Siwisa N, Thomas K, Der Walt M. Extensively Drug-Resistant TB in Eastern Cape, South Africa: High Mortality in HIV-Negative and HIV-Positive Patients. *J Acquir Immune Defic Syndr* 2011; 57(2):146.
2. World Health Organization. Global Tuberculosis Report. Geneva, Switzerland: World Health Organization; 2015 p. 1-3. [28 February 2017]. Available from: http://www.who.int/tb/publications/global_report/en/
3. Iseman MD. Tuberculosis therapy: past, present and future. *Eur Respir J Suppl* 2002 Jul;36:87s-94s
4. Donald PR, Diacon AH. Para-aminosalicylica acid: the return of an old friend. *Lancet Infect Dis* 2015; 3:1091-1099.
5. Peloquin CA, Henshaw TL, Huitt GA, Berning SA, Nitta AT, James GT. Pharmacokinetic evaluation of para-aminosalicylic acid in patients with drug-resistant pulmonary tuberculosis. *Pharmacotherapy* 1994; 14:40-46.
6. Kanabus Annabel. Information about tuberculosis. 2017 [30 March 2017] Available from: http://www.tbfacts.org/drug-resistant-tb-south-africa/.
7. Lehmann J. The role of the metabolism of p-aminosalicylic acid (PAS) in the treatment of tuberculosis. Interaction with the metabolism of isonicotinic acid hydrazide (INH) and the synthesis of cholesterol. *Scand J Respir Dis* 1969; 50:169-185.
8. PASER® GRANULES 2016-last update. Aminosalicylic acid granule, delayed release [Package insert]. Princeton, New Jersey [Homepage of Jacobus Pharmaceutical Company, Inc]. 1996 [30 March 2017]. Available: http://www.americanpharmawholesale.com/store.php/AmericanPharmaWholesale/pd9055497
9. Cynamon MH. *Tuberculosis: current concepts and treatment "Chemotherapeutic agents for mycobacterial interactions*, Second ed. New York : CRC Press LLC ; 2001
10. Momekov G, Momekova D, Stavrakov G, Voynikov Y. Para-aminosalicylic aid - Biopharmaceutical, pharmacological, and clinical features and resurgence as an anti-tuberculous agent . *Pharmacia* 2015; 62(1): 25-51.
11. Berning SE, Peloquin CA. *Antimycobacterial agents: p-Aminosalicylic acid.* , First ed. Baltimore: Williams and Wilkins; 1998.
12. Otten H, Piempel M, Siegenthaler W. Antibiotika-Fibel, 4th ed. Stuttgart: Georg Thieme Verlag; 1975.

13. Wang P, Pradhan K, Zhong X, Ma X. Isoniazid metabolism and hepatotoxicity. *Acta Pharmaceutica Sinica B* 2016; 6(5): 384-392.
14. Butcher N, Boukouvala S, Sim E, Minchin R. Pharmacogenetics of the arylamine N-acetyltransferases. *The pharmacogenomics journal* 2002; 2(1): 384-392.
15. Meyer UA. Polymorphism of human acetyltransferases. *Environ Health Perspect* 1994; 102 (Suppl 6): 213-216.
16. Sim E, Lack N, Wang CJ, Long H, Westwood I, Fullam E . Arylamine N-acetyltransferases: structural and functional implications of polymorphisms. *Toxicology* 2008; 254(3): 170-183.
17. Blum M, Grant D, McBride W, Heim M, Meyer UA. Human arylamine N-acetyltransferase genes: Isolation, chromosomal localization, and functional expression. *DNA and cell biology* 1990; 9(3): 193-203.
18. Badenhorst CPS, van der Sluis R, Erasmus E, van Dijk A. Glycine conjugation: Importance in metabolism, the role of glycine-N-acyltransferase and factors that influence interindividual variation. *Expert Opin. Drug Metab. Toxicol*. 2013; [Early Online] 1-15.
19. Knights KM, Sykes MJ, Miners JO. Amino acid conjugation: contribution to the metabolism and toxicity of xenobiotic carboxylic acids. *Expert Opin. Drug Metab. Toxicol.* 2007; 3(2): 159-168.
20. Lipmann F. *J Biol Chem* 1945; 160:173.
21. Dubovsky H. Correspondence with a pioneer, Jurgen Lehmann (1898-1989). *S Afr Med* 1991; 79: 48-50.
22. Lehmann J. Para-aminosalicylic acid in the treatment of tuberculosis. *Lancet* 1946; 1: 15.
23. Lauener H, Favez G. The inhibition of isoniazid inactivation by means of PAS and benzoyl-PAS in man. *Am Rev Respir Dis* 1959; 80:26-37.
24. Johnson W, Corte G. The inhibition of isoniazid acetylation in vitro and vivo. *Proc Soc Exp Biol Med* 1956; 92:446-448.
25. Morse WC, Curry FJ, Morse PZ, Chambers JS, Lincoln AF. Transactions of the 15th Conference on the Chemotherapy of tuberculosis. February 5th to 9th, 1956. St. Louis, Missouri, Washington DC: US Government Printing Office; 1956.
26. Lehmann J. The treatment of tuberculosis in Sweden with para-aminosalicylic acid; a review. *Dis Chest* 1949; 16:684-703.

27. Medical Research Council. Treatment of pulmonary tuberculosis with isoniazid; an interim report to the Medical Research Council by their tuberculosis Chemotherapy Trials Committee. *BMJ* 1952; 2:735-746.
28. Marsden JA. The single daily dose of sodium para-aminosalicylic acid used in conjunction with streptomycin in tuberculosis. *Med J Aust* 1954; 2:934-935.
29. Riska N. Variations in the PAS concentration in the blood and their influence on treatment of tuberculosis. *Acta Tuberc Pneumol Scand* 1954; 30:144-163.
30. Riska N. PAS therapy with a daily unfractionated dose. *Acta Tuberc Pneumol Scand* 1959; 37:104-111.
31. Riska N, Tennberg C. Optimal PAS dosage. *Am Rev Respir Dis*1962; 86:430-433.
32. Donald PR, Schaaf HS. Old and new drugs for the treatment of tuberculosis in children. *Paediatric respiratory reviews* 2007; 8(2):134-141.
33. Peloquin CA, Berning SE, Huitt GA, Childs JM, Singleton MD, James GT. Once-daily and twice-daily dosing of p-aminosalicylic acid granules. *Am J Respir Crit Care Med* 1999; 159:932-934.
34. Sy SKB, de Kock L, Diacon AD et al. N-acetyltransferase genotypes and the pharmacokinetics and tolerability of para-aminosalicylic acid in patients with drug-resistant pulmonary tuberculosis. *Antimicrob Agents Chemother* 2015; 59:4129-4138.
35. Jindani A, Aber VR, Edwards EA, Mitchison DA. The early bactericidal activity of drugs in patients with pulmonary tuberculosis. *Am Rev Respir Dis* 1980; 121:939-949.
36. Daniels M, Hill AB. Chemotherapy of pulmonary tuberculosis in young adults; an analysis of the combined results of three Medical Research Council trials. *BMJ* 1952; 1:1162-1168.
37. Donald PR, Diacon AH. Para-aminosalicylica acid: the return of an old friend. *Lancet Infect Dis* 2015; 3:1091-1099.